“Affective-related endophenotypes in serotonin transporter over-expressing mice”

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Declaration of Originality

I declare that the composition of this thesis and the work presented herein is my own, unless otherwise stated. This work has not been and is not concurrently submitted for any other degree.

Abstract

The affective disorders (anxiety and depression) are common psychiatric disorders that primarily involve disturbances in mood and represent the second leading source of disease burden world-wide. A wide base of evidence supports a significant genetic contribution to these disorders. Polymorphic variation in the promoter region (5-HTTLPR) of the human serotonin transporter (hSERT) gene, which leads to a life-long alteration in serotonin transporter (SERT) expression and functioning, has been implicated in the aetiology of both anxiety and depression. Despite the strong evidence implicating a role for this polymorphism in affective psychopathology the underlying mechanism by which genetically determined SERT bioavailability influences affective functioning are not understood.

In these studies I attempt to elucidate the alterations in cerebral, serotonin (5-HT) system and hypothalamo-pituitary-adrenal (HPA) axis functioning which may relate to the effect of the 5-HTTLPR on affective functioning by characterising these parameters in an animal model of genetically increased SERT expression (*h*SERT over-expressing mice; *h*SERT OVR). Furthermore, as gender influences both the likelihood of developing affective disorders and the impact of the 5-HTTLPR on affective functioning, with a greater effect being observed in females than in males, we characterise these parameters in mice of both genders.

The data presented in this thesis demonstrate that the life-long increase in SERT bioavailability present in *h*SERT OVR mice produces profound alterations in cerebral, serotonin system and HPA axis functioning. Furthermore, the influence of increased SERT expression upon cerebral and serotonin system functioning is greater in females than in males. Additionally, a number of sexually dimorphic variations in serotonin system functioning were identified. Thus this thesis extends the currently available information regarding the underlying mechanisms by which gender and a life-long alteration in SERT expression may influence the risk of affective psychopathology.

List of Publications arising from thesis

Dawson N, Ferrington L, Olverman HJ, Harmar AJ, Kelly PAT. (2005) Altered cerebral glucose metabolism in the response to DOI in serotonin transporter over-expressing mice. Proceedings of the British Pharmacological Society at http://www.pA2online.org/abstracts/Vol3Issue4abst166P.pdf

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Abbreviations

[^{14}C]-2-DG	2-deoxy-D-glucose-1-^{14}C
[^{14}C]-UR	[^{14}C]-uptake ratio analysis
2-DG-P	2-deoxy-D-glucose-6-phosphate
5,7-DHT	5,7-dihydroxytryptamine
5-HIAA	5-hydroxyindolacetic acid
5-HT	5-hydroxytryptamine; serotonin
5-HTTLPR	5-HTT gene-linked polymorphic regions
5-HTP	5-hydroxy tryptophan
8-OH-DPAT	8-hydroxy-2-(di-*n*-propylamino)tetralin
AC	adenylate cyclase
ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
ADH	aldehyde dehydrogenase
ADHD	attention deficit hyperactivity disorder
AR	aldehyde reductase
ATP	adenosine trisphosphate
AVP	arginine vasopressin
BBB	blood brain barrier
BPD	bipolar disorder
Ca2+	calcium
cAMP	cyclic adenosine monophosphate
Citalopram	1-[3-(dimethylamino)propyl)-1-(4-fluorophenyl)-1,3-dihydro-5-isobenzofurancarbonitrile
CNS	central nervous system
CP 94,253	5-propoxy-3-{1,2,3,6-tetrahydro-4-pyrinyl)-1H-pyrrolo[3,2-b}pyridine
CP 93,129	1,4-Dihydro-3-(1,2,3,6-tetrahydro-4-pyridinyl)-5H-pyrrolo[3,2-b]pyridine-5-one
CRF/CRH	corticotrophin releasing factor/hormone
CSF	cerebrospinal fluid
DA	dopamine
DAG	diacylglycerol
DAT	dopamine transporter
dNTPs	deoxyribonucleotide trisphosphates

DOI	1-(4-iodo-2,5-dimethoxy-phenyl)propan-2-amine
DRN	dorsal raphé nucleus
DST	dexamethasone suppression test
Elisa	enzyme-linked immunosorbent assay
GABA	gamma aminobutyric acid
GAD	generalized anxiety disorder
GPCR	G-protein coupled receptor
GR	glucocorticoid receptor
HPA	hypothalamo-pituitary-adrenal axis
HIOMT	5-hydroxylindole o-methyl transferase
*h*SERT OVR	human serotonin transporter over-expressing
IP_3	inositol trisphosphate
i.p.	intraperitoneal
ISH	in situ hybridisation
i.v.	intravenous
Kd	dissociation constant
Ketanserin	3-[2-[4-Fluorobenzoyl)-1-piperidinyl]ethyl]-2,4[1H,3H]-quinazolinedione
KO	knock-out
LCMRglu	local cerebral glucose utilisation
LGIC	ligand gated ion channel
LSD	lysergic acid diethylamide
MAO	monoamine oxidase
MAOI	monoamine oxidase inhibitors
MAPK	mitogen-activated protein kinase
MDD	major depressive disorder
MDMA	methylenedioxymethamphetamine
Mesulergine	N'-(1,6-Dimethylergolin-8alpha-yl)-N,N-dimethylsulfamide
mPFC	medial prefrontal cortex
MR	mineralocorticoid receptor
MRN	median raphé nucleus
mRNA	messenger ribonucleic acid
NA	noradrenaline
NAT	noradrenaline transporter
NO	nitric oxide

NSB	non-specific binding
OCD	obsessive compulsive disorder
OD	optical density
Paroxetine	3-[(1,3-Benzodioxol-5-yloxy)methyl]-4-(4-fluorophenyl)-piperidine
PCR	polymerase chain reaction
PD	panic disorder
PTSD	post-traumatic stress disorder
PKC	protein kinase C
PKG	protein kinase G
POMC	pro-opiomelanocortin
PVN	paraventicular nucleus
ROI	region of interest
SAD	social anxiety disorder
SCN	suprachiasmatic nucleus
SERT	serotonin transporter
SNAT	5-hydroxytryptamine N-acetyl transferase
SNP	single nucleotide polymorphism
SQ 2-DG	semi-quantitative 2-deoxyglucose autoradiography
SSRI	selective serotonin reuptake inhibitor
TB	total binding
TCAs	tricyclic antidepressants
TMB	tetramethylbenzidine
TMD	transmembrane domain
WAY 100,635	N-[2-[4-(2-Methoxyphenyl)-1-pi-perazinyl]ethyl]-N-2-pyridinylcyclohexanecarboxamide
Wt	wild-type
VMAT	vesicular monoamine transporter
VNTR	variable nucleotide tandem repeat sequence

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Chapter 1 – Introduction

1. Affective disorders: Epidemiology and Aetiology

Affective disorders are common psychiatric disorders that primarily involve disturbances in mood. They are a major cause of disability and represent the second largest source of global disease burden (Collier et al., 1996). The most common affective disorder major depressive disorder (MDD, or unipolar depression) has a lifetime risk of between 15-20% in the general population with women having twice (~21%) the prevalence of men (~12%) (Blazer et al., 1994; Fava abd Kendler, 2000). MDD is characterised by a major depressive episode lasting two or more weeks with symptoms such as depressed mood, anhedonia, disturbed sleep, inability to concentrate, psychomotor alterations and suicidal thoughts. In contrast to MDD, bipolar disorder (BPD, manic depression) is characterised by depressive episodes with intermittent periods of extreme elation, often accompanied by psychotic disturbances. The lifetime risk of BPD at approximately 1% is lower than that of MDD (Rees et al., 1997). The anxiety disorders (panic disorder (PD), social anxiety disorder (SAD), generalized anxiety disorder (GAD), obsessive-compulsive disorder (OCD) and post-traumatic stress disorder (PTSD)) which are characterised by excessive fearfulness are also common (~4% lifetime risk of any anxiety disorder). Again these disorders are more prevalent in females than in males (~5% v ~2%) (Schuckit et al., 1997) and anxiety disorders also display a high level of co-morbidity with the depressive disorders being present in ~11% to ~65% of depressed patients (Pini et al., 1997). The dramatic impact of depressive and anxiety disorders on the individual sufferer, their families and society as a whole emphasises the importance of further elucidating the underlying neurobiology and physiology of these disorders in order to improve treatment and possibly develop prevention strategies.

Although affective disorders are clinically heterogeneous with complex aetiologies there is considerable evidence in support of there being a significant genetic component in the development of these disorders. For example, studies have shown that the concordance of MDD between genetically identical, monozygotic twins is around 50% (Oswald et al., 2004) and meta-analysis suggests that the overall heritability of MDD is approximately 33% (Sullivan et al., 2000). Genetic factors also appear to be important in BPD where data show a 75-80% risk of developing the disorder in the co-twins of affected monozygotic twins, as compared to the 1% risk in the general population (Rees et al., 1997). Similarly, meta-analysis of anxiety disorders indicates that their estimated heritability is approximately 40%-50% (Hettema et al., 2001).

Genome-wide linkage surveys have only recently been applied to identify the chromosomal loci of the genes that are involved in depressive and anxiety disorders (Boomsa et al., 1999; Nash et al., 2004; Zubenko et al., 2003). However, as affective disorders are highly multigenic in their aetiology, with each gene contributing a relative small increase in the risk, the power of linkage analysis studies to detect genetic effects is limited. An alternative approach has been to use genetic association studies to investigate the possible role of genetic variants (polymorphisms) in affective disorders, and a number of these have been completed. Because of the proposed central role for the monoamines in affective functioning (Bunney Davis, 1965; Carlsson et al., 1969; Lapin and Oxencrug, 1969), these association studies have largely focused on polymorphisms present in those genes involved in the regulation of monoaminergic neurotransmission. To date a role for several monoaminergic-related genetic polymorphisms have been identified in the aetiology of the affective disorders, but despite the high co-morbidity of anxiety and depressive disorders few studies have identified a role for the same genetic polymorphism in both of these disorder types. Nevertheless, there is compelling evidence in support of a role for polymorphic variation in the promoter region (5-HTTLPR) of the serotonin transporter gene, which leads to a life-long alteration in serotonin transporter (SERT) expression and function, in the aetiology of both anxiety and depressive disorders (see section 6.3). However, despite this strong evidence implicating the 5-HTTLPR in affective disorders, the exact mechanistic link between genetically-regulated SERT activity and affective functioning is not understood, but it is likely to involve changes to the central serotonergic system.

In this thesis I have examined indices of activity in the central serotonin (5-HT) system and hypothalamo-pituitary-adrenal (HPA) axis in an animal model of genetically increased SERT function. The meaningful interpretation of these data in relation to the effect that the 5-HTTLPR may have upon on affective function in the human brain requires a sound understanding of the anatomy of the central 5-HT system, the physiological and pharmacological processes of 5-HT neurotransmission, and the functional roles of 5-HT, with particular attention paid to the involvement of the 5-HT system in psychopathology and control of HPA axis functioning.

2. Neuroanatomy of Central Serotonergic System

Twarog and Page (1953) were the first to detail the presence of 5-HT in the CNS, noting also that concentrations of 5-HT varied between different brain areas. The anatomical localisation of the cell bodies and terminal fields of 5-HT-containing neurones within the CNS (rat) was not detailed, however, until over 10 years later following the development of fluorescent histochemical techniques (Dahlstrom and Fuxe, 1964a,b; 1965). These original classifications identified nine discrete clusters (B1 to B9) of 5-HT-containing cell bodies in the raphé region of the midbrain and were found to be analogous to those previously identified some 54 years earlier by Ramon y Cajal (1911) as "large multipolar neurones with unknown projections". It was later shown that the 5-HT system consisted of two subdivisions, with the most caudal groups of cells in the medulla (B1-B3) giving rise to axons that descend to innervate the ventral and dorsal horns and the lateral spinal cord, while the more rostral groupings in the dorsal and median raphé project to the forebrain (Ungerste, 1971). More recently the anatomy of the 5-HT system has been more precisely characterised using both autoradiographic and immunohistochemical methods (Riad et al., 2001; Steinbusch et al., 1981) and these studies have indicated that this neurotransmitter system represents the most expansive and complex neurochemical system in the mammalian CNS.

2.1 Ascending (Rostral) Serotonergic Projections

Cell bodies of the rostrally-projecting 5-HT system are found in the dorsal (DRN; B6 and B7) and median raphé nuclei (MRN; B5 and B8), the caudal linear nucleus (CLN; B8) and group B9 (Jacobs and Azmitia, 1992; Tork 1990). Six ascending pathways from these nuclei innervate anatomically and functionally diverse regions of the forebrain, including many components of the limbic system. The DRN gives rise to four pathways (the arcuate, cortical, periventricular and forebrain tracts) whose targets include neocortex, basal ganglia, thalamic and hypothalamic nuclei, amygdala and the suprachiasmatic nucleus (SCN). Two pathways arise from the MRN (median raphé forebrain tract and raphé medial tract) that innervate the SCN, preoptic area, olfactory bulb, hippocampus, entopeduncular nucleus and the mamillary body. Despite its close proximity to the MRN the efferents of the CLN most closely match those of the DRN (Imai et al., 1986). Although the DRN and MRN each preferentially innervate different forebrain regions, their anatomical arrangement is such that most areas of the brain receive input from both. Moreover, it must also be noted that 5-HT neurones are highly collateralised which means that a single 5-HT neurone may innervate several

projection fields (Deolmos and Heimer, 1980; Vanderkooy and Hattori, 1980; Waterhouse et al., 1986) and this anatomical arrangement allows the potential for 5-HT neurones to co-ordinate the activity of several functionally-related nuclei. In addition to the differences in the topographic distribution of innervation between the DRN and MRN the fibres arising from these two nuclei are also morphologically distinct. DRN (D) fibres have fine axons (<1µM diameter) with irregularly spaced varicosities that branch frequently, whereas, MRN (M) fibres are thin and have large spherical variscosities (typically 2-5µM diameter) (Kohler et al., 1980; Kosofsky and Molliver, 1987; Mamounas and Molliver, 1988; Mulligan and Tork, 1988). It is the ascending 5-HT systems arising from the DRN and MRN that are of primary interest in this thesis.

Figure 1.1 Serotonin System Neuroanatomy

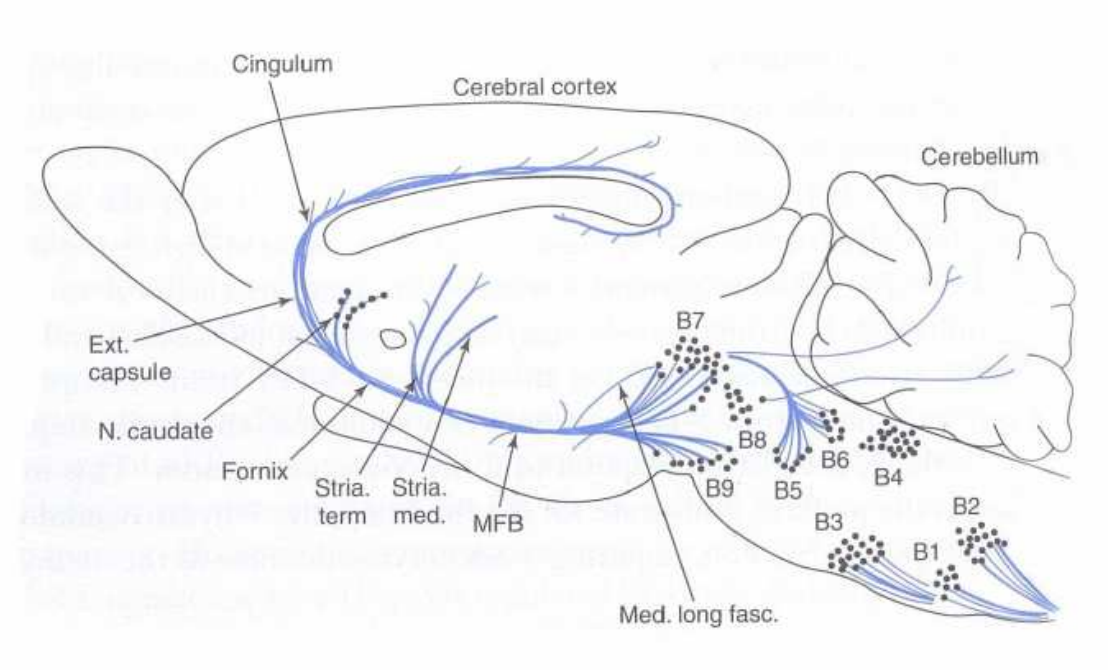


Diagram illustrating the distribution of the major clusters of serotonergic neurones and pathways in the rat CNS. Ext. capsule= external capsule, N. caudate = caudate nucleus, Stria. term= stria terminalis, MFB= median forebrain bundle. (Derived from Cooper et al, 2003).

2.2 Descending (Caudal) Serotonin Projections

5-HT cells with their cell bodies in the nucleus raphé pallidus (NRPa), nucleus raphé obscurus (NRO) and the nucleus raphé magnus (NRM), corresponding to B1,B2 and B3 respectively, give rise to the descending 5-HT system (Dahlstrom and Fuxe, 1964). 5-HT fibres of the descending system innervate both sensory and motor nuclei of the autonomic system at every spinal level. A more detailed review of the descending system, however, is out-with the scope of this thesis as the involvement of these systems in the following studies is extremely limited. Jacobs and Azmitia (1992) provide a comprehensive review detailing descending serotonergic neuroanatomy.

2.3 Afferent Innervation of Raphé Nuclei

The activity of raphé neurones is modulated by a number of neurotransmitters and neuromodulatory peptides, and the raphé nuclei receive a range of afferent input from a number of other neurotransmitter systems. The most extensive innervation of the raphé nuclei, however, comes from the nuclei themselves. Thus, extensive connections have previously been shown to link the DRN, MRN and B9 cell groups to each other (Mosko et al., 1977; Stratford and Wirtshafter, 1988). Innervation of the raphé by the other monoaminergic neurotransmitter systems has also been found, with afferents from the locus coeruleus providing noradrenergic (NA) innervation (Aghajanian and Wang, 1977; Swanson and Hartman, 1975) and afferents from the substantia nigra and ventral tegmental area (VTA) providing dopaminergic (DA) innervation to the raphé (Kalen et al., 1988; Lira et al., 2003). These innervations provide neuroanatomical evidence of the reciprocal interactions that exist between 5-HT and the other neurotransmitter systems as all of these regions receive dense innervation from 5-HT raphé neurones.

Interestingly, an increasing body of evidence suggests that the raphé receive functional afferent inputs from a number of other forebrain regions, including afferents from the medial prefrontal cortex (mPFC) (Peyron et al., 1998; Sesack et al., 1989). These afferents appear to activate GABAergic interneurones localised within the raphé resulting in the inhibition of 5-HT neuronal firing (Hajos et al., 1999; Varga et al 2001; 2003). A similar mechanism of action has been proposed for afferent innervation of the raphé arising in the lateral habenula (Herkenham and Nauta, 1979; Varga et al., 2003; Wang and Aghajanian, 1977).

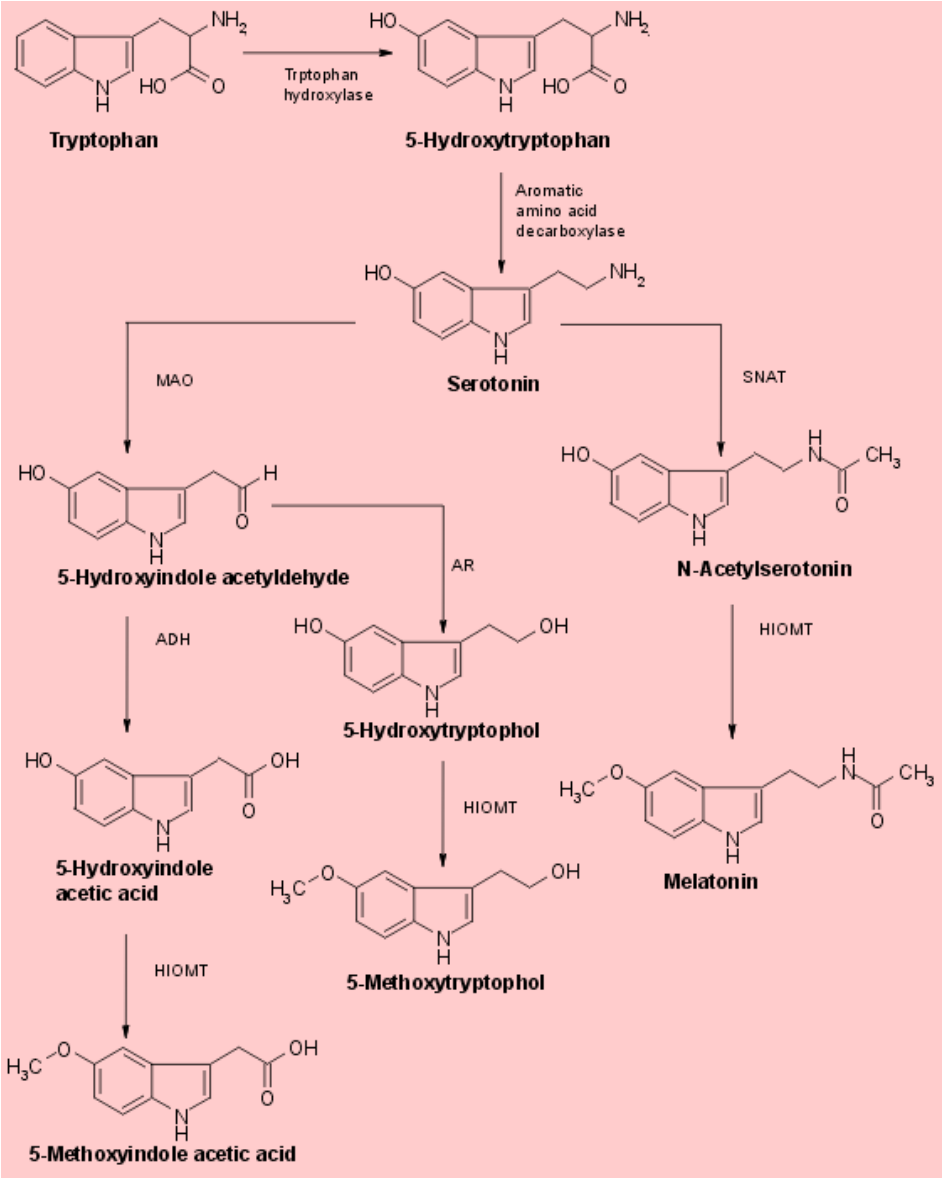
3. Serotonergic Neurotransmission

3.1 Biosynthesis and Metabolism of Serotonin

5-HT is synthesised in a two step reaction from the essential dietary amino acid L-tryptophan and availability of this amino acid is rate-limiting in 5-HT synthesis. L-tryptophan is transported across the blood brain barrier (BBB) by the large neutral amino acid carrier, responsible for the transport of all of the large neutral amino acids into the CNS (tyrosine, leucine, isoleucine, valine and phenylalanine). Competition between these amino acids for the transporter, therefore, determines L-trytophan availability in the CNS. In neurones the 5-position of the indole ring of L-tryptophan is hydroxylated by the cytoplasmic enzyme tryptophan hydroxylase, which is specifically expressed only in 5-HT neurones (Lovernberg et al., 1968). The resulting 5-hydroxytrytophan (5-HTP) from this reaction is then rapidly decarboxylated by non-specific L-aromatic amino acid decarboxylase, forming 5-HT.

In most cells 5-HT is metabolised by monoamine oxidase (MAO) located on the mitochondrial outer membrane. MOA-A, one of two isoforms of the enzyme, deaminates 5-HT to the intermediate 5-hydroxyindole acetaldehyde. This is then metabolised by NAD^+-sensitive aldehyde reductase to form the major metabolite 5-hydroxyindole acetic acid (5-HIAA). Further metabolism of 5-HIAA to 5-hydroxytryptanol can also occur, although this accounts for only 1% of 5-HT metabolite levels present in the brain (Cheifetz and Warsh, 1980). Additionally, alternative metabolic pathways can result in the production of other neuroactive substances from 5-HT. For example, melatonin is synthesised from 5-HT in the pineal gland (Klein and Weller, 1970) and evidence also suggests that 5-HT may be biotransformed by cytochrome P450 resulting in the generation of nitric oxide (NO) (Fradette et al., 2004).

Figure 1.2 Synthesis and Metabolism of Serotonin



Abbreviations: Monoamine oxidase (MAO), Aldehyde reductase (AR), Aldehyde dehydrogenase (ADH), 5-hydroxylindole 0-methyltransferase (HIOMT), 5-hydroxytryptamine N-acetyl transferase (SNAT). Adapted from Joh et al., 1998.

3.2 Storage and Release

Following neuronal synthesis, 5-HT from the cytoplasm is sequestered into terminal storage vesicles by the vesicular monoamine transporter (VMAT). Of the two VMAT isoforms (VMAT1 and VMAT2) VMAT2 is predominantly responsible for vesicular transport of all monoamines in the CNS, having similar affinities for NA, DA and 5-HT (Liu and Edwards, 1997; Masson et al., 1999). Vesicular transport of 5-HT is driven by the electrochemical gradient created as a result of vesicular H^+ ion uptake by an ATP-driven pump. In the process of 5-HT translocation two internalised H^+ ions are exchanged for one molecule of cytoplasmic 5-HT by VMAT (Parsons, 2000; Schuldiner, 1994). Within the vesicle 5-HT is bound by a specific binding protein which increases the concentration of 5-HT that can be stored in the vesicle.

The arrival of an action potential at the nerve terminal causes 5-HT release from vesicular stores by Ca^{2+}-dependent exocytosis. A review of the molecular mechanisms of this process are out-with the scope of this thesis but several comprehensive reviews are available (Burgoyne and Cheek, 1995; Sollner, 2003). Once released into the extracellular space 5-HT activates a number of post-synaptic and pre-synaptic 5-HT receptors.

3.3 Serotonin Receptors

In 1957 Gaddum and Picarelli first identified two pharmacologically distinct 5-HT receptors, termed M and D, responsible for the 5-HT-induced contractile response of the guinea pig ileum. Over 20 years later subtypes of the 5-HT receptors were further confirmed using radioligand-binding studies (Peroutka and Snyder, 1979). The first attempt to classify 5-HT receptors was completed by Bradley et al. (1986) on the basis of receptor pharmacology and the functional responses elicited by the receptors. These studies led to the identification of three families of 5-HT receptor (5-HT_{1-3}), comprising five distinct receptor binding sites. With some prescience the authors suggested that the future identification of other receptor subtypes was highly likely. Of the three families, the 5-HT_1 receptor subtype were found to represent a heterogeneous group of receptors at which 5-HT displayed high affinity, while 5-HT_2 receptors were identified as being analogous to the D receptor and 5-HT_3 receptors were analogous to the M receptor previously identified by Gaddum and Picarelli (1957).

Since this initial classification advances in radioligand-binding and molecular biological techniques have lead to the discovery of many more 5-HT receptor subtypes. At present these are classified into seven receptor families (5-HT_{1-7}) compromising a total of 14 structurally and pharmacologically distinct receptor subtypes (Hoyer et al., 2002). Comprehensive reviews of all known 5-HT receptor subtypes are available (Barnes and Sharp, 1999; Hoyer et al., 1994), and Table 1 provides a brief summary of the CNS distribution of each receptor subtype, its effector mechanism, behavioural functions and the neurochemical effects of receptor activation. A more detailed analysis of the pharmacology and function of every 5-HT receptor subtype is out with the scope of this thesis, but those receptors with a putative role in the psychopathology of affective disorders, namely 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A} and 5-HT_{2C} receptors will be considered in more detail.

Table 1.1 Serotonin Receptor Subtypes

Receptor	Mechanism	CNS high density	Behavioural Functions	Neurotransmitter Effects					
				5-HT	NA	ACh	Glut	DA	GABA
5-HT_{1A}	GPCR,Gi/G ↓AC	Raphé, HP, Septum, CTX	Anx., Dep., Aggr., Loco., Thermo., Hyperphagia	↓[a]	↑	↑	↓	↑	↓
5-HT_{1B}		SNR, GP, DS	Anx., Dep., Aggr., Sex., Loco., Rotation, Hypophagia, Migr.	↓[a]	↓	↓	↓		↓
5-HT_{1D}		SNR, GP	Anx., Dep., Migr.	↓[a]			↓		↓
5-HT_{1E}		CTX, HP, Caud., Amygd.	Unknown, lack of selective ligands						
5-HT_{1F}		CTX, HP, Caud.	Migr.	Unknown, lack of selective ligands					
5-HT_{2A}	GPCR, Gαs ↑ PLC	N.Acc., HP, CTX, Caud.	Anx., Dep., Apetite, Thermo., Sleep, Hallucination	↓	↓	↓		↓	↓
5-HT_{2B}		CTX, Sept, Amyg., Hypo.	Anx.						
5-HT_{2C}		Choroid plexus, N.Acc., Amyg., HP	Anx., Dep., Sleep, C.V., Loco., Migr.	↓	↓			↓	↓
5-HT_{2D}		HP	Unknown, lack of selective ligands						
5-HT_{3}	LGIC	Entorhinal CTX, Dorsal Vagal Complex	Anx., Loco., Reward	↑	↓	↓		↑	↑
5-HT_{4}	GPCR, Gs ↑ AC	HP, Colliculi, Mesolimbic, Nigrostriatal	Anx., Cogn., Loco.	↑		↑		↑	
5-HT_{6}	GPCR, Gs ↑ AC	Striatum, N. Acc., Basal Ganglia	Motor, Anx., Dep., Cogn.			↑			
5-HT_{7}	GPCR, Gs ↑ AC	Thal., Hypo., Amyg., SCN	Anx., Dep., Learning and Memory, Circadian rhythm, Sleep	↓		↓			

Overview of 5-HT receptor subtype localisation, behavioural functions and neurochemical effects. ↑indicates increased activity/availability whereas ↓indicates decreased activity/availability. Abbreviations: AC: adenylate cyclase, Amyg: amygdala, Anx: anxiety, Caud: caudate, CTX: cortex, Cogn: cognitive, C.V: cardiovascular, Dep: depression, DS: dorsal subiculum, GP: globus Pallidus, GPCR: G-protein coupled receptor, HP: hippocampus, Hypo: hypothalamus, LGIC: ligand gated ion channel, Loco: locomotion, Migr: migraine, N.Acc: nucleus accumbens, PLC: phospholipase C, SCN: suprachiasmatic nucleus, SNR: substantia nigra pars reticulata, Thermo: thermoregulation. [a] *denotes autoreceptor effect. Developed from (Barnes and Sharp, 1999; Bonaventure et al., 2007; Bonsi et al., 2007; Diaz-Mataix et al., 2005; Hirst et al., 2003; Matsuyama et al., 1997; Nelson, 2005).*

3.4 5-HT$_1$ Receptor Family

The 5-HT$_1$ receptor family consists of five receptors subtypes (5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1D}$, 5-HT$_{1E}$ and 5-HT$_{1F}$) defined by their high degree of amino acid sequence homology. All of these receptors are G-protein coupled receptors (GPCRs) that couple negatively to adenylate cyclase (AC) via pertussis toxin-sensitive G-proteins (G_i/G_o). Therefore, activation of these receptors results in decreased intracellular cyclic adenosine-3',5'-monophosphate (cAMP) levels.

3.4.1 5-HT$_{1A}$ Receptors

Following identification of two distinct 5-HT$_1$ binding sites, termed 5-HT$_{1A}$ and 5-HT$_{1B}$ (Middlemiss and Fozard, 1983; Pedigo et al., 1981), understanding of the pharmacology and function of the 5-HT$_{1A}$ receptor subtype progressed quickly due in no small part to the development of the selective 5-HT$_{1A}$ agonist 8-OH-DPAT and synthesis of its tritiated analogue ([^{3}H]-8-OH-DPAT), although it is now apparent that 8-OH-DPAT also displays moderate affinity for the 5-HT$_7$ receptor subtype (Lovernberg et al., 1968; Shen et al., 1993). A number of selective 5-HT$_{1A}$ antagonists, of which WAY 100,635 is the most selective, have also been developed (Johansson et al., 1997), and both radiolabelled [^{3}H]-8-OH-DPAT and [^{3}H]-WAY 100,635 have been used with autoradiographic imaging to map the distribution of the 5-HT$_{1A}$ receptor in the mammalian CNS (Castro et al., 2003; Khawaja, 1995). The highest density of 5-HT$_{1A}$ binding sites are found in limbic brain regions, particularly in subregions of the hippocampus, the lateral septum, cortical regions (including cingulate and entorhinal) and also the dorsal raphé. Lower levels are found in the median raphé, amygdala regions and some hypothalamic and thalamic nuclei. These findings have been replicated by *in vivo* binding studies in humans, primates and mice with few differences in the pattern of distribution reported between species (Farde et al., 1997; Laporte et al., 1994; Pike et al., 1995).

5-HT$_{1A}$ receptors are predominantly somatodendritic and they are located post-synaptically in forebrain areas receiving 5-HT projections and as autoreceptors on raphé 5-HT neurones (Kia et al., 1996; Miquel et al., 1991; Pompeiano et al., 1992). In forebrain regions 5-HT$_{1A}$ receptor expression has been detected in cortical pyramidal (glutamatergic) neurones, hippocampal granular and pyramidal (glutamatergic) neurones and cholinergic neurones in the septum (Burnet et al., 1995). 5-HT$_{1A}$ receptors are also expressed by corticotrophin

releasing factor-containing (CRF) neurones in the paraventricular nucleus (PVN) of the hypothalamus, an area which contributes to the regulation of neuroendocrine components of the stress response (see section 5.2). In addition, 5-HT_{1A} receptors are localised on 5-HT cell bodies and processes and GABAergic interneurones in the raphé (Day et al., 2004). The somatodendritic localisation of autoreceptors on 5-HT raphé neurones has been confirmed by the observation that levels of raphé 5-HT_{1A} binding sites are maintained following 5,7-dihydroxytryptamine (5,7-DHT) lesion, which is selectively neurotoxic to 5-HT nerve terminals (Radja et al., 1991). Activation of these somatodendritic 5-HT_{1A} autoreceptors by extracellular 5-HT inhibits raphé efferents and leads to a reduction in the synaptic release of 5-HT in the forebrain. In addition to this local mechanism of 5-HT_{1A}-receptor-mediated negative feed-back on 5-HT neuronal activity, evidence also suggests that "long-loop" negative feed-back mechanisms exist that also involve the post-synaptic 5-HT_{1A} receptor. For example, activation of post-synaptic 5-HT_{1A} receptors in the medial prefrontal cortex is proposed to inhibit the activity of glutamatergic (excitatory) neurones which provide reciprocal, descending innervation of the raphé. Inhibition of this normally excitatory innervation of the raphé is proposed to result in decreased 5-HT neuronal activity (Celada et al., 2001; Hajos et al., 1999).

In addition to their neuronal localisation there is also evidence that 5-HT_{1A} receptors are expressed by astroglial cells (Whitaker-Azmitia et al., 1993). At present the function of these glial 5-HT_{1A} receptors is unknown. However, the localisation of 5-HT_{1A} receptors to glia of the hippocampal dentate gyrus (DG) and the finding that 5-HT_{1A} receptor stimulation promoters the secretion of pro-survival factors by glia (Jacobs and Azmitia, 1992), can activate pro-survival signalling cascades (Cowen, 2007) and stimulates neurogenesis and progenitor survival in this region (Huang and Herbert, 2005) suggests a putitative role for glial 5-HT_{1A} receptors in the regulation of hippocampal neurogenesis.

Neuronal 5-HT_{1A} receptors signal via coupling to G_i/G_o proteins to inhibit cAMP formation, inactivate calcium channels and activate potassium channels (Lemonde et al., 2003). These actions are inhibitory, reducing neuronal activity and neurotransmitter release. However, in hippocampal tissue, positive coupling to adenylate cyclase has also been reported (Markstein et al., 1986; Shenker et al., 1983) although this may ultimately be attributed to pharmacological activation of the 5-HT_7 receptor. Furthermore, even if positive coupling to adenylate cyclase does occur in hippocampal neurones then it would appear that the negative coupling mechanisms of 5-HT_{1A} receptor signalling may predominate. Electrophysiology

studies confirm that post-synaptic 5-HT_{1A} receptor activation does indeed decreases neuronal activity in the hippocampus, in part as a result of potassium channel activation (Graeff et al., 1996; Tada et al., 1999). Further support for the importance of non-cAMP mediated signalling mechanisms in 5-HT_{1A}-receptor mediated inhibition of neuronal activity is evident from studies on raphé 5-HT neurones. 5-HT_{1A} receptor activation does not alter cAMP levels in raphé membranes (Clarke et al., 1996) but isolated DR neurones have been shown to display inwardly rectifying potassium currents and an inhibition of calcium currents in response to 5-HT_{1A} autoreceptor activation (Lemonde et al., 2003; Penington et al., 1991; 1993; Williams et al., 1988).

The pre- and post-synaptic localisation of 5-HT_{1A} receptors and their diverse neuroanatomical localisation contribute to the wide-spread alterations in extracellular neurotransmitter levels observed following their activation and also the diverse behavioural and physiological functions associated with this receptor (See table 1). The relative importance of pre- versus post-synaptic 5-HT_{1A} receptors is often unclear and highly contentious, although there is good evidence that both are implicated in anxiety and depression. Thus, there are many studies that support a role for reduced post-synaptic 5-HT_{1A} receptors expression, binding and function in depression (Adlersberg et al., 2000; Drevets et al., 2000; Lopez-Figueroa et al., 2004; Sargent et al., 2000), while an important role for altered 5-HT_{1A} autoreceptor function in depression is also supported by observations of increased 5-HT_{1A} autoreceptor binging in depressed patients (Stockmeier et al., 1998). Furthermore, the selective desensitisation of 5-HT_{1A} autoreceptors by chronic antidepressant treatment has been proposed as central to their therapeutic effect in depressed patients (Hervas et al., 2001; Lemonde et al., 2003; Riad et al., 2001). Despite a relative paucity of data investigating 5-HT1A binding in anxiety diosorders patients decreased post-synaptic and autoreceptor binding has been reported in both PD and social anxiety disorder (Lanzenberger et al., 2005; Neumeister et al., 2004). A role for the 5-HT_{1A} receptor in the regulation of affective functioning is also supported by evidence from preclinical studies showing that animals in which 5-HT_{1A} function is ablated (5-HT_{1A} receptor knock-out mice) display increased anxiety-like behaviour (Heisler et al., 1998; Zhuang et al., 1999; Ramboz et al., 2002). As conditional rescue of 5-HT_{1A} receptor function in the forebrain of these animals during the early postnatal period, but not in adult-hood, is able to reverse the anxiogenic effect of 5-HT_{1A} receptor knock-out (Gross et al., 2002) this suggests that post-synatpic 5-HT_{1A} receptors may play an important role in regulating anxiety through developmental mechanisms. In addition to this developmental role the level of 5-HT_{1A}

receptor activation in the adult brain also appears to regulate anxiety with a differential role for the autoreceptors (anxiolytic) and post-synaptic (hippocampal, anxiogenic) receptors supported (File et al., 1996).

3.4.2 5-HT_{1B} Receptors

The 5-HT_{1B} receptor subtype was originally identified in rodent brain tissue as a [^{3}H]-5-HT binding site with low affinity for spiperone (Pedigo et al., 1981). Later, the observation that this binding site also displayed low affinity for the specific 5-HT_{1A} agonist 8-OH-DPAT further established the pharmacological difference between the two receptors. It was originally thought that the 5-HT_{1B} receptor was species-specific to rodents (rat, mouse, hamster) (Hamon et al., 1986; Pedigo et al., 1981). However, further pharmacological characterisation and sequencing studies have shown that rodent 5-HT_{1B} receptor is homologous to the human 5-$HT_{1D\beta}$ receptor (Adham et al., 1992). The same nomenclature is now recommended for this receptor subtype in all mammalian species with the human 5-$HT_{1D\beta}$ receptor now known as h5-HT_{1B} and the rodent as r5-HT_{1B} (Hartig et al., 1996; Sari, 2004). Distinct pharmacological differences between these receptor homologues, such as higher affinity of r5-HT_{1B} for (-)propranolol than the h5-HT_{1B}, are the result of a single amino-acid difference between these receptors (Hamblin et al., 1992; Metcalf et al., 1992) in which there is otherwise 97% homology.

A number of radioligands have been used in order to map the distribution of the 5-HT_{1B} receptor in autoradiographic studies. These include the use of non-specific ligands such as [^{3}H]-5-HT in the presence of blocking concentrations of 5-HT_{1A} and 5-HT_{2C} ligands (Peroutka, 1986) and more recently the use of more 5-HT_{1B} selective ligands such as [^{3}H]CP 93,129 (Koe et al., 1992) and [^{3}H]GR 125,743 (Varnas et al., 2001). In such studies a high density of 5-HT_{1B} binding has been identified in basal ganglion regions, including the substantia nigra, globus pallidus (GP) and entopeduncular nucleus in the rodent brain. These results have been confirmed, and the distribution of the 5-HT_{1B} receptor expanded, by use of antibodies specific to the receptor. In these studies high levels of the 5-HT_{1B} receptor were also confirmed in the dorsal subiculum (DS), with lower levels identified in the caudate-putamen, cerebral cortex and peri-aqueductal grey (PAG) (Sari, 2004).

Both radioligand binding and immunocytochemical techniques have demonstrated that 5-HT_{1B} receptors localise to axons and axon terminals (Boulenguez et al., 1996; Sari, 2004).

An increasing body of evidence suggests that 5-HT_{1B} receptors function as both autoreceptors modulating the release of 5-HT from 5-HT nerve terminals (Buhlen et al., 1996; Middlemiss, 1990) and heteroreceptors on non-serotonergic terminals regulating the release of other neurotransmitters including glutamate (Li and Bayliss, 1998), GABA (Johnson et al., 1992; Stanford and Lacey, 1996) and acetylcholine (Maura and Raiteri, 1986). 5-HT_{1B} receptors are GPCRs that mediate their inhibitory effect on neurotransmitter release by coupling negatively to adenylate cyclase via G_i/G_o proteins (Sari, 2004).

A role for the 5-HT_{1B} receptor has been suggested in multiple physiological functions, behaviours and psychiatric disorders including locomotion, migraine, drug abuse reinforcement and aggression. A wealth of evidence from preclinical studies also suggests a role for 5-HT_{1B} receptors in anxiety, depression and the response to antidepressant treatment. For example, 5-HT_{1B} knock-out mice display reduced anxiety-like behaviour in a number of paradigms (Brunner et al., 1999; Zhaung et al., 1999; Malleret et al., 1999). Furthermore, pharmacological activation of 5-HT_{1B} receptors is anxiogenic (Lin and Parsons, 2002) and also produces antidepressant-like effects (O'Neil; et al., 1996; O'Neill and Conway, 2001). With regard to antidepressant treatment, a down-regulation or desensitisation of 5-HT_{1B} autoreceptors is proposed to be central to their therapeutic effect and the combined administration of 5-HT_{1B} antagonist with SSRI antidepressants may potentiate their efficacy (Sari, 2004). Despite considerable preclinical evidence implicating 5-HT_{1B} in anxiety and depression there is a paucity of data on CNS binding and function from clinical studies. This may be due to the lack of selective 5-HT_{1B} ligands available until recently. However, one study has reported an increase in 5-HT_{1B} receptor mRNA levels in the hippocampus of depressed suicide victims (Lopez-Figueroa et al., 2004), suggesting that 5-HT_{1B} may be altered in depression.

3.5 The 5-HT_2 Receptor Family

The 5-HT_2 receptor family consists of three receptor subtypes (5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}) which possess very similar molecular structures and couple to the same signal transduction pathways. All 5-HT_2 receptors stimulate phospholipase C (PLC) activity via $G_{\alpha s}$ protein activation resulting in an increased accumulation of the second messangers inositol phosphate (IP_3) and diacylglycerol (DAG), which in turn lead to increased intracellular calcium and increased protein kinase C (PKC) activity respectively (Barnes and Sharp, 1999).

3.5.1 5-HT_{2A} Receptors

The 5-HT_{2A} receptor is analogous to the D receptor previously defined by Gaddum and Picarelli (1957) in the guinea pig ileum. In the CNS this receptor subtype also corresponds to the classical 5-HT_2 binding site defined by Peroutka and Synder (1979) as having high affinity for [^{3}H]spiperone and [^{3}H]LSD but a low affinity for [^{3}H]5-HT. The use of [^{3}H]spiperone and the discovery, and subsequent tritiation, of the selective 5-HT_{2A} antagonist ketanserin which displays >1000 fold selectivity for the 5-HT_{2A} over the 5-HT_{2B} and 5-HT_{2C} receptors, were central in mapping the distribution of this receptor in the CNS (Castro et al., 2003; Leysen et al., 1982). A number of other radiolabelled ligands including [^{125}I]DOI and more recently [^{3}H]MDL 100,907 have also been used to map 5-HT_{2A} receptor distribution (Li et al., 2003; Lopez-Gimenez et al., 1997). These studies show that 5-HT_{2A} receptors are expressed widely throughout the rodent CNS including the caudate nucleus, nucleus accumbens, the hippocampus and many cortical regions. The distribution of this receptor in rodents is closely matched in humans, as detected by [^{11}C]MDL 100,907 in PET and other ligands in post-mortem studies (Forutan et al., 2002; Hinz et al., 2007; Pandey et al., 2002). The high concordance between 5-HT_{2A} binding sites, immunoreactivity and the mRNA distribution for the receptor in the CNS suggests that the receptors are located post-synaptically. Indeed, 5-HT_{2A} expression has been identified on cortical interneurones (GABAergic) (Burnet et al., 1995; Lira et al., 2003; Morilak et al., 1993) and pyramidal (glutamatergic) neurones (Wright et al., 1995).

5-HT_{2A} activation results in excitatory neuronal responses in a variety of brain regions including the cortex and hippocampus. This has been shown electrophysiologically in cortical neurones by the application of the 5-HT_{2A} agonists DOI and LSD (Marek and Aghajanian, 1996; 1999) and by the blockade of 5-HT-evoked neuronal activation by the specific 5-HT_{2A} antagonist MDL 100,907 in both the cortex and hippocampus (Marek and Aghajanian, 1994; Piguet and Galvan, 1994). Alterations in potassium conductance are, in part, responsible for these depolarisations but the contribution of the phosphoinositide signalling pathway in this effect is not certain (Aghajanian et al., 1990).

Initially, a central role for the 5-HT_2 receptors in depression and anxiety disorders was supported by the finding of altered 5-HT_2 receptor binding (with non-selective 5-HT_2 ligands) in the brains of depressed suicide victims (Arango et al., 1992; Cheetham et al., 1988; Yates et al., 1990). More recently, due to the availability of more selective ligands, 5-

HT_{2A} receptor binding and function have been shown to be altered not only in post-mortem brains of depressed suicide victims (Rosel et al., 2004) but also in the living brains of depressed patients (Mintun et al., 2004; Sheline et al., 2004) and OCD sufferers (Adams et al., 2005). In preclinical studies the anxiolytic-like effects of the mixed 5-$HT_{2A/2C}$ agonist DOI have also been shown to be mediated specifically by the 5-HT_{2A} receptor (Dhonnchadha et al., 2003a). In addition, mice lacking the 5-HT2A receptor (genetic knock-out) display decreased anxiety-like behaviour in conflict paradigms, an effect which can be reversed by the cortical rescue of 5-HT_{2A} receptor function during early brain development (Weisstaub et al., 2006). These results suggest that cortical 5-HT_{2A} receptors may play a role in determining anxiety through developmental mechanisms. Overall the pre-clinical and clinical data support the suggestion that 5-HT_{2A} receptors play a role in both anxiety and depressive disorders.

3.5.2 5-HT_{2C} Receptors

The 5-HT_{2C} receptor was initially identified as a binding site in the choroid plexus that could be labelled by [^{3}H]mesulergine and [^{3}H]LSD but not by [^{3}H]ketanaserin (Castro et al., 2003). Autoradiographic studies have utilised a number of radioligands, including [^{3}H]LSD, [^{3}H]mesulergine and [^{125}I]DOI to characterise the distribution of 5-HT_{2C} receptors in the CNS. In addition to the very high levels of expression detected in the choroid plexus 5-HT_{2C} receptors are widely distributed in areas of limbic system (nucleus accumbens, amygdala, hippocampus), the basal ganglia (caudate nucleus, substantia nigra) and many cortical regions (Castro et al., 2003; Li et al., 2003; Radja et al., 1991). In general there is good concordance between the distribution of 5-HT_{2C} mRNA expression and 5-HT_{2C} binding sites and immunohistochemistry (Mengod et al., 1990; Sharma et al., 1997). These observations, along with the fact that 5-HT_{2C} immunoreactivity is upregulated following 5,7-DHT lesion, supports a post-synaptic localisation for the 5-HT_{2C} receptor. However, in the lateral habenula 5-HT_{2C} mRNA has been detected whereas 5-HT_{2C} binding in this region is very low suggesting a possible pre-synaptic location for these receptors on lateral habenula projections (Barnes and Sharp, 1999). This has yet to be confirmed.

In line with the functional responses of the other 5-HT_2 receptors, 5-HT_{2C} receptors have been shown to increase phopholipase C activity via a G-protein dependent mechanisms (Boess and Martin, 1994; Sandersbush et al., 1988). This mechanism may contribute to the excitatory role of the 5-HT_{2C} receptor on neurones in several brain regions. 5-HT_{2C} receptors

have, for example, been shown to directly excite cortical pyramidal cells (Sheldon and Aghajanian, 1991) and to be involved in the 5-HT mediated excitation of GABAergic (dorsal raphé) (Boothman et al., 2003) and cholinergic interneurones (Bonsi et al., 2007)

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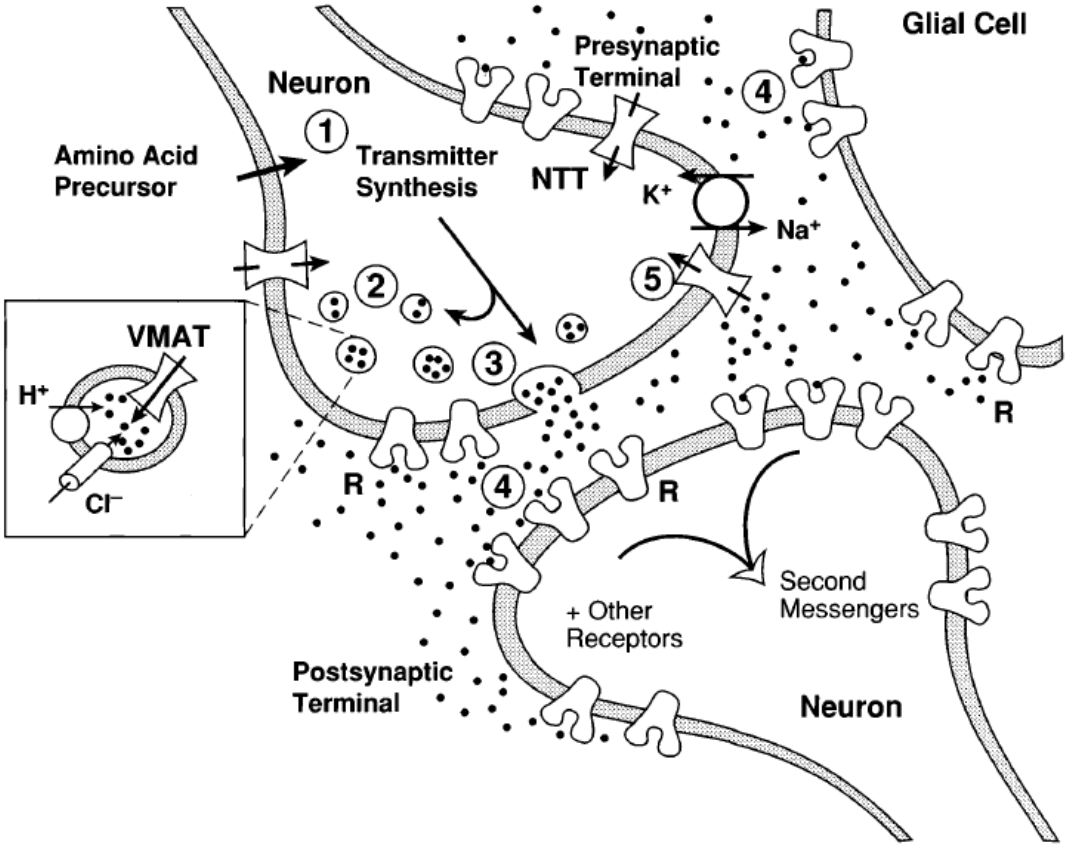
Evidence supporting a role for the 5-HT_{2C} receptor subtype in depression and anxiety largely comes from preclinical studies showing that antagonists of the receptor are anxiolytic (Kuznetsova et al., 2006; Martin et al., 2002) and that the anxiogenic-like effects of mCPP in mice are blocked by administration of 5-HT_{2C} antagonists (Hackler et al., 2007). In the future, as mCPP is also known to be anxiogenic in humans, co-administration of specific 5-HT_{2C} antagonists with mCPP may further confirm the role of the 5-HT_{2C} receptors in anxiety. The role of the 5-HT_{2C} receptor in anxiety is also supported by the observation that animals in which 5-HT_{2C} function is ablated, by genetic knock out, display decreased anxiety-related behaviours (Heisler et al., 1999; Heisler et al., 2007; Tecott et al., 1996). A role for the 5-HT_{2C} receptor in determining compulsive behaviour is also supported by observations made in 5-HT_{2C} receptor knock-out mice (Chou-Green et al., 2003). To date there are no studies assessing the binding levels or function of central 5-HT_{2C} receptors in anxiety disorder or depressed patients. However, recent analysis of 5-HT_{2C} pre-mRNA in the post-mortem brains of suicide victims suggests that pre-mRNA editing of 5-HT_{2C} mRNA was altered in the brains of suicide victims in such a way as to alter the efficacy of 5-HT_{2C} signalling (Gurevich et al., 2002b). In the future it is likely that studies in humans will investigate both the possibility of altered 5-HT_{2C} binding and signalling in depression and anxiety.

Figure 1.3 Serotonergic Neurotransmission



Schematic representation of components of neurotransmission at a serotonergic synapse. (1) 5-HT (solid circles) is synthesised from its amino acid precursor (tryptophan). (2) 5-HT is then packaged into vesicles by VMAT. (3) 5-HT is released into the synaptic cleft following depolarisation of the presynaptic neurone. (4) Extracellular 5-HT activates pre- and post-synaptic neuronal 5-HT receptors and possibly glial 5-HT receptors. (5) 5-HT is cleared from the synapse by the serotonin transporter (SERT). In the cytoplasm 5-HT is either re-packaged into vesicles or metabolised by monoamine oxidase (MAO). Note the presence of the Na+/K+ ATPase in the plasma membrane, which maintains the ionic gradients used to drive 5-HT reuptake by SERT. Also note on the enlarge synaptic vesicle the H+-ATPase which provides the driving force for 5-HT transport into the vesicle. Figure from Hoffman et al., 1998.

3.6 Serotonin Reuptake

The activity of 5-HT at its receptors is terminated by its removal from the synaptic cleft. Reuptake of 5-HT into the presynaptic terminal by the serotonin transporter (SERT) is the principle mechanism by which 5-HT is removed from the cleft. SERT belongs to a family of structurally and pharmacologically distinguishable transporter proteins known as the plasma membrane monoamine transporters (Amara and Kuhar, 1993; Torres et al., 2003). Other members of this family include the dopamine transporter (DAT) and the noradrenaline transporter (NAT), responsible for the reuptake of DA and NA respectively.

3.6.1 The Serotonin Transporter (SERT)

SERT is made up of 630 amino acids arranged into 12 α-helical transmembrane domains (TMDs) with a predicted 5 intracellular and 6 extracellular loops with both the carboxy- and amino-termini located intracellularly (Masson et al., 1999; Rudnick and Clark, 1993). Multiple sites for N-linked glycosylation are located on the large extracellular loop between TMDs 3 and 4, whereas consensus sequences for possible phosphorylation by a range of kinases exist at different intracellular sites (Hoffman et al., 1998). N-glycosylation of the transporter appears to be essential for the transport and cell surface expression of the transporter while the phosphorylation state of SERT plays a role in regulating the availability of the transporter at the membrane to allow 5-HT reuptake (Blakely et al., 1998; Qian et al., 1997). Although most models of transporter function have assumed that transporters function as single units, an increasing body of evidence suggests that SERT may function as an oligomer as it displays activity as both a dimer (2 units) and tetramer (4 units)(Jess et al., 1996; Kilic and Rudnick, 2000; Qu et al., 2003).

Inwardly-directed Na^+ and Cl^- gradients and outwardly directed H^+ and K^+ gradients provide the driving force for 5-HT transport across the plasma membrane by SERT. These electrochemical gradients are created and maintained by the plasma membrane Na^+/K^+ ATPase (Kanner and Schuldiner, 1987; Rudnick and Clark, 1993) and allow for the accumulation of 5-HT concentrations in the nerve terminal to several hundred-fold that observed in the extracellular space. The ionic dependence has been established and 5-HT transport by SERT involves the internalisation of one Na^+ and one Cl^- ion with the externalisation of one K^+ ion (Gu et al., 1994). This means that 5-HT transport is electrochemically neutral. However, in addition to this substrate transport mechanism it is

suggested that the monoamine transporters also exhibit channel-like activity as they all demonstrate ionic conductances that can not be accounted for by the fixed stoichiometry of substrate translocation (Galli et al., 1996; Mager et al., 1994; Sonders et al., 1997).

3.6.2 SERT Localisation

Characterisation of the anatomical and cellular localisation of SERT in the CNS has been possible due to the availability of specific radioligands for the transporter, including [^{3}H]paroxetine and [^{3}H]citalopram, the development of SERT antibodies and the advent of RNA sequencing, which has allowed for detection of SERT mRNA expression (Qian et al., 1995; Sur et al., 1996). There is good concurrence between SERT expression as detected by radioligand autoradiography and immunocytochemistry. SERT is present and functional on the surface of every component of the 5-HT neurone including dendrites, peikarya, axons and nerve terminals. Evidence also suggests that SERT may be expressed and functional on glial cells (Bel et al., 1997; Inazu et al., 2001), which may also play an important role in the regulation of extracellular 5-HT availability. SERT mRNA expression is highest in the raphé nuclei with lower levels detectable in some projection terminal fields such as the cortex, hippocampus and striatum but there are clear parallels between SERT mRNA expression in regions outside the raphé and high densities of serotonergic innervation suggesting that SERT mRNA detected in these regions may be associated with 5-HT neurones.

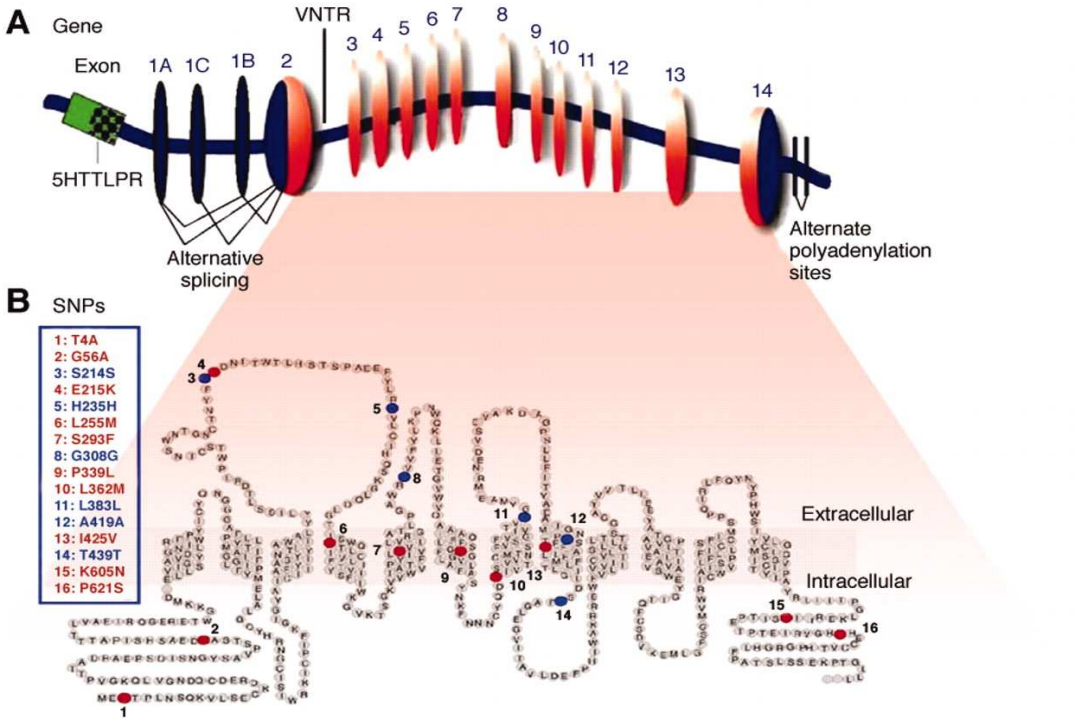
3.6.3 Regulation of SERT function

SERT expression and function is regulated by multiple factors at different levels, from that of gene transcription to post-translational modification. Despite increasing knowledge of the mechanisms involved in the regulation of SERT the full nature of these mechanisms is yet to be fully elucidated.

Regulation of SERT function at the genetic level is produced by coding variations present in the SERT gene. To date several single nucleotide polymorphisms (SNPs) have been identified in the human SERT gene (*h*SERT) (Cargill et al., 1999). Recently two non-synonymous SNPs, nucleotide codons that code for different amino acid insertions, were found to alter basal 5-HT transport capacity by altering the expression levels of SERT (Kilic et al., 2003; Prasad et al., 2005). Furthermore, five other SNPs were found to alter the regulatory effects of protein kinase G (PKG) and p38 mitogen-activated protein kinase

(MAPK) signalling on SERT functioning (Prasad et al., 2005). However, two other polymorphisms in the human SERT gene have received the most attention because they have been directly implicating in affective disorders. One, a variable nucleotide tandem repeat sequence (VNTR) in intron 2 of the SERT gene confers higher transcriptional activity when 12 copies of the VNTR are present in comparison to lower copy numbers (Fiskerstrand et al., 1999; MacKenzie and Quinn, 1999). The second polymorphism, a common promoter variant in the 5' flanking region located ~1.4kb upstream of the transcription start site, termed the 5-HTT gene-linked polymorphic region (5-HTTLPR) also confers higher expression and SERT function when composed of sixteen ("long" or "L" allele, 44 base pair insertion) as compared to fourteen ("short" or "S" allele, 44 base pair deletion) repeated elements (Greenberg et al., 1999; Lesch et al., 1996; Little et al., 1998).

Figure 1.5 Organisation of the SERT gene and protein



Schematic diagram showing organisation of the human SERT gene and protein. (A) Diagram of SERT gene organisation showing coding exons (red) and non-coding introns (blue). Note the location of the 5-HTTLPR upstream of the first exon and the location of the VNTR near exon 2. (B) Schematic of amino acid sequence in SERT protein with amino acids coded by single nucleotide polymorphisms (SNPs) in the gene shown. Some result in altered amino acids (red) insertions while others (blue) do not. Note also the 12 transmembrane helices, internal carboxy and amino termini and intracellular and extracellular loops. Figure from Murphy et al (2004).

In addition to the chronic regulation of SERT function dictated by the polymorphisms present in the *h*SERT gene, evidence also supports a role for several mechanisms involved in the acute regulation of SERT functioning, including those that regulate gene transcription and post-translational modification. For example, chronic elevation of cAMP has been shown to increase SERT-encoding mRNA (Ramamoorthy et al., 1993) suggesting that signalling pathways linked to intracellular cAMP may play a role in the regulation of SERT function by altering the rate of transcription. Theoretically, this mechanism could provide a link between 5-HT autoreceptor activation and alterations in SERT transcription. In turn, this pathway may play a part in the observed transcriptional regulation of SERT function following chronic antidepressant treatment, which decreases SERT-encoding mRNA expression (Lesch, 1993). However, others have found no effect of chronic antidepressant treatment on SERT transcription (Benmansour et al., 1999). Modified SERT functioning resulting from transcriptional alterations are likely to occur after a significant time delay (hours). However, evidence also suggests that SERT function can be regulated over a shorter time frame by mechanisms involving the activation of multiple GPCRs, including the 5-HT_{1B} receptor (Daws et al., 2000), as well as by several protein-kinase and protein-phosphatase linked pathways. Several studies have shown that activation of protein kinase C (PKC) and protein kinase G (PKG), or inhibition of protein phosphatase 2Ac (PP2Ac) and p38 mitogen-activated protein kinase (MAPK), decreases SERT activity in a number of systems (Jayanthi et al., 1994; Prasad et al., 2005; Qian et al., 1997; Ramamoorthy and Blakely, 1999; Samuvel et al., 2005). Evidence from PKC studies largely suggests that the phosphorylation state of SERT dictates whether it is expressed at the cell surface or whether it is redistributed to intracellular compartments (Ramamoorthy and Blakely, 1999; Torres et al., 2003). However, the observation that down-regulation of the transporter by PKC activation is not prevented by removal of the PKC consensus phosphorylation sites from SERT suggests that direct phosphorylation of SERT may not be the primary mechanism of SERT regulation by PKC (Sakai et al., 1997). In contrast, modification of SERT function by PKG is dependent upon the direct phosphorylation of threonine residue 276 in SERT (Ramamoorthy et al., 2007) and the effect of MAPK and PKG activation on SERT function is altered by genetically determined variation in the amino acid sequence of SERT (Prasad et al., 2005). These findings suggest that the regulation of SERT by different protein-phosphorylation and protein-phosphatase linked pathways is likely to involve diverse mechanisms. Additionally, protein-kinase linked pathways may be central to the acute regulation of SERT function by extracellular 5-HT levels, as down-regulation of SERT by PKC activity is sensitive to 5-HT (Ramamoorthy and Blakely, 1999) suggesting a possible

integrative mechanism for regulating SERT function in response to both intracellular and extracellular conditions.

4. Functional Roles of Serotonin

In the CNS 5-HT neurones innervate components of all functional systems and regulate the neuronal activity of sensory, motor, autonomic and enteric neurones. Therefore 5-HT is implicated in a diverse range of functions including appetite, sleep, learning and memory, temperature regulation, motor behaviour, endocrine regulation and affective functioning.

4.1 Psychopathology of Serotonin

Due to its involvement in the regulation of numerous functional systems and behaviours, dysfunction of 5-HT neurotransmission has been implicated in a multitude of psychopathologies including aggression, impulsivity, schizophrenia, obsessive compulsive disorder (OCD), attention deficit hyperactivity disorder (ADHD) and Autism. A prominent role has also been suggested in a number of neurodegenerative diseases such as Alzheimers and Parkinsons. However, most research to date has been dedicated to further elucidating the role of 5-HT in the affective (depression and anxiety) disorders.

The monoamine hypothesis of depression was initially put forward to explain the antidepressant effects of iproniazid, a monamine amine oxidase inhibitor (MAOIs), and imipramine, a tricyclic antidepressant (TCA) (Bunney and Davis, 1965; Schildkraut, 1965). The hypothesis suggested that depression resulted from a functional deficit in monoaminergic (5-HT and/or NA) neurotransmission in key brain areas while mania was the result of a functional excess of transmission. Therefore, TCAs and MAOIs were proposed to elicit their antidepressant effect by increasing extracellular monoamine concentrations. This hypothesis stimulated much research into the role of these monoamines in depressive disorders and has dominated the biological approach to depression since its proposal. Later, the observation that the ranked affinity of imipramine-related drugs for the inhibition of 5-HT reuptake correlated well with their mood-elevating efficacy, lead to the suggestion that 5-HT may be the most important neurotransmitter in the antidepressant effects of TCAs (Carlsson et al., 1969). This knowledge, along with the observation that 5-HT and 5-HIAA concentrations are reduced in both the cerebrospinal fluid (CSF) of depressed patients (Ashcroft et al., 1966; Dencker et al., 1966) and the post-mortem brains of depressed suicide

victims (Pare et al., 1969; Shaw et al., 1967) lead to the development of the indoleamine hypothesis of depression (Carlsson et al., 1969; Lapin and Oxenkrug, 1969), placing even more importance on 5-HT in depressive disorders. At this time the indolemine hypothesis was also supported by the observation that supplementing the diet of depressed patients with the amino acid precursor for 5-HT, tryptophan, was also antidepressant (Coppen et al., 1967; Hertz and Sulman, 1968). To date a multitude of studies have also reported on the depressogenic effects of acute tryptophan depletion, a dietry challenge which decreases central 5-HT neurotransmission, in depression susceptible individuals (Klaassen et al., 1999; Moreno et al., 1999) and remitted depressed patients (Leyton et al., 2000; Moreno et al., 1999). It was the development of the selective serotonin re-uptake inhibitors (SSRIs) such as paroxetine and citalopram in the 1980s and their establishment as highly effective antidepressants, however, that truly cemented a central role for 5-HT in the aetiology depression.

Although there is much evidence supporting a role for the monoamine and indoleamine hypotheses in depression they do not explain the reason for the delay in the mood elevating effects of TCA, MAOI or SSRI treatment in comparison to their rapid enhancement of extracellular monoamine levels. Furthermore, these hypotheses do not explain the effectiveness of antidepressants in the treatment of anxiety disorders (Vaswani et al., 2003; Zohar and Westenberg, 2000). In contrast to the proposed mechanism of 5-HT function in depression, 5-HT is classically viewed as being anxiogenic (Iversen, 1984; Wise et al., 1972) and anxiety disorders are thought to be associated with enhanced 5-HT neurotransmission. As anxiety and depression often display co-morbidity, both being present in the same patient, it is hard to reconcile the two conflicting hypothesis of 5-HT functioning in these disorders. It has been suggested, however, that these differences may be due to the involvement of differential serotonin receptor subtypes and/or neural substrates in these disorders (Graeff et al., 1996).

Despite the conflicting theoretical role for 5-HT in depressive and anxiety disorders, however, there remains considerable evidence for convergent alterations (genetic and molecular) in the 5-HT system in both of these disorders. For example, functional polymorphisms in a number of 5-HT receptor gene subtypes have been found to increase the risk of developing both anxiety and depressive disorders (5-HT_{1A}: Lemonde et al., 2003; Maron et al., 2005, 5-HT_{2A}: Bonnier et al., 2002; Gorwood et al., 2002; Inada et al., 2003; Little et al., 1998, 5-HT_{2C}: Gutierrez et al., 1998; Lerer et al., 2001). Furthermore, consistent

alterations in 5-HT_{1A} receptor binding have been reported in both anxious and depressed patients. Post-synaptic 5-HT_{1A} binding has been reported to be decreased in depression (Drevets et al., 1999; Lesch et al., 1992b; Lopez et al., 1998) panic disorder (Neumeister et al., 2004) and social anxiety disorder (Lanzenberger et al., 2005). In contrast 5-HT_{1A} autoreceptor function has been reported to be increased in depression and panic disorder (Drevets et al., 1999; Neumeister et al., 2004b; Stockmeier et al., 1998). In addition to 5-HT_{1A} receptor findings a decrease in the density of SERT binding has been reported in the brains of both living depressed patients (Bremner et al., 1997; Staley et al., 1998) and those suffering from panic disorder (Maron et al., 2003). However, while SERT density was found to be reduced on the platelets of patients with GAD (Hernandez et al., 2002; Iny et al., 1994) reduced binding in the brain was not confirmed (Maron et al., 2004). These data suggest that common genetic and molecular events in the 5-HT system play a role in both anxiety and depressive disorders. However, future research dedicated to elucidating alterations in the other receptor subtypes in both anxiety and depressive disorders is required. From the studies already completed, however, it is interesting to note that while alterations in 5-HT_{1A} and SERT binding are consistent in directionality in both anxiety and depression the neuroanatomical localisation of these alterations is different. These findings give some credence to the proposal by Graeff et al. (1996) that alterations in differential neural networks may be central to depression and anxiety.

Extensive pre-clinical data also support a role for 5-HT in the regulation of affective functioning as modification of 5-HT system functioning results in altered affective-related behaviours in animal models. While pharmacological challenge studies targeting specific 5-HT receptor subtypes have been informative on the regulation of affective-related behaviours for some of the 5-HT receptor subtypes, a lack of specific ligands for other receptor subtypes has limited the usefulness of pharmacological studies in determining the role of these receptors in affective behaviour. Studies in transgenic animals with targeted disruption (knock-out; KO) of specific 5-HT receptor subtypes, however, have been particularly useful in investigating the role of specific 5-HT receptor subtypes in affective-related behaviours. Serotonin receptor KO animals have been used to investigate the role of the specific 5-HT receptor subtypes investigated in this thesis in affective-related behaviours (see relevant study introduction sections for more detailed information). In addition, evidence from transgenic animals also supports a role for the 5-HT_3 (Bhatnagar et al., 2004) and 5-HT_7 (Guscott et al., 2005; Hedlund et al., 2005) in depressive and/or anxiety-related behaviours. The availability of animals with targeted disruption of the other 5-HT receptor subtypes,

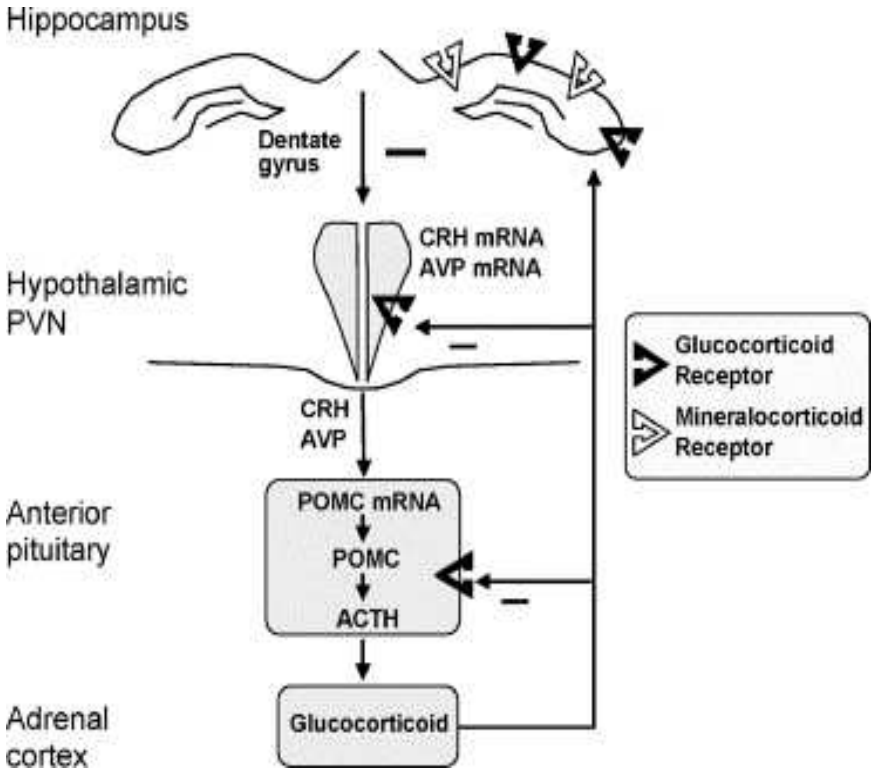
including the 5-HT_4 (Conductier et al., 2006) and 5-HT_6 receptor (Bonasera et al., 2006) subtypes, may allow for the further elucidation of the possible role of these receptors in affective functioning. Furthermore, producing conditional KO animals for the specific 5-HT receptor subtypes in the future may be able to further elucidate the relative importance of the developmental role versus the adult level of 5-HT receptor functioning in determining affective-related behaviour. In this way, the use of 5-HT_{1A} receptor conditional KO animals has already shown that the developmental mechanisms regulated by cortical 5-HT_{1A} receptors may be particularly important in determining anxiety-related behaviours (Gross et al., 2002).

4.2 5-HT regulation of hypothalamo-pituitary-adrenal (HPA) axis function

4.2.1 Anatomy of the hypothalmo-pituitary-adrenal (HPA) axis

Activation of the hypothalamo-pituitary-adrenal (HPA) axis is a major component of the stress response and leads to behavioural and physiological adaptations that attempt to restore homeostasis and ensure survival. Parvocellular neurones of the paraventricular nucleus (PVN) located in the hypothalamus are responsible for the release of corticotrophin releasing hormone/factor (CRH/ CRF) and arginine-vasopressin (AVP) at the median eminence into the portal system of the anterior pituitary. Activation of CRF receptors on corticotroph cells present in the anterior pituitary causes them to secrete adrenocorticotropic hormone (ACTH) into the peripheral blood system. Simultaneous activation of AVP receptors on the same cells acts synergistically to enhance CRH secretion while AVP alone has little ACTH secretagogue activity (Lamberts et al., 1984; Liu et al., 1983). At the adrenal cortex circulating ACTH regulates the secretion of glucocorticoids (cortisol in humans, corticosterone in rodents) into the circulation. Glucocorticoids are the final effectors of the HPA axis and are involved in a multitude of responses that contribute to the control of whole body homeostasis. Under non-stressful conditions CRH and AVP are secreted into the portal system in a pulsatile fashion with a frequency of approximately 3 secretory episodes per hour (Engler et al., 1989b). This secretion also displays a circadian rhythm, with the amplitude of CRH and AVP secretions being greater upon waking resulting in higher ACTH and cortisol concentrations (Antoni, 1986; Horrocks et al., 1990). During stressful experiences the rate and amplitude of pulsatile CRH and AVP secretion is increased (Charmandari et al., 2005) resulting in increased circulating glucocorticoid levels.

Figure 1.6 Anatomy of the HPA axis



Schematic of the hypothalamo-pituitary-adrenal (HPA) axis. Parvocellular neurones of the PVN produce CRF and AVP. These factors stimulate the synthesis and release of ACTH from anterior pituitary corticotroph cells into the systemic circulatory system. At the adrenal cortex ACTH simulates the release of glucocortiocoids. Glucocortioids act at multiple loci within the body to maintain homeostasis and also act in the CNS to modify behaviour. In the CNS glucocorticoids also act at glucocorticoid (GR) and mineralocorticoid (MR) receptors to inhibit HPA axis activity in a classical negative feed-back loop. Figure from Owen et al., 2005.

Glucocorticoids exert their affects through their ubiquitous cytoplasmic receptors, the mineralocorticoid (MR; type I) and glucorticoid (GR; type II) receptors. Within the CNS MR expression is highest within the septo-hippocampal (McEwen et al., 1986) complex whereas the GR receptor is more widely distributed with high levels present in the dentate gyrus, lateral septum, PVN, the thalamus and cortical regions (DeKloet et al., 1984). When bound to their ligands these receptors translocate to the nucleus where they form homodimers to interact with specific glucocorticoid-responsive elements (GREs) present within the DNA to activate the transcription of specific hormone-responsive genes (Pratt, 1990). In addition to this classical genomic pathway evidence also suggests that rapid, non-genomic mechanisms also play a part in mediating the more immediate effects of glucocorticoids. For example, the rapid effect of corticosterone on neuronal activity is proposed to involve possible alterations in membrane properties by non-genomic mechanisms governed by the classical receptors or by activation of a putative G-protein linked membrane receptor (Tasker et al., 2005; 2006).

Glucocortiocoids also play a central role in the regulation of HPA axis activity by a negative feedback mechanism, acting at extrahypothalamic sites (such as the hippocampus), the hypothalamic PVN and pituitary gland to limit the secretion of CRH and ACTH. Occupancy of the MR receptor, which has a very high affinity for the glucocorticoids, is involved in the tonic control of corticosterone secretion. GR occupancy is involved in termination of the stress axis response when high levels of glucocorticoids are present, as this receptor has lower affinity for glucocortiocoids and is only significantly occupied at higher glucocorticoid concentrations (DeKloet and Reul, 1987; Reul et al., 2000).

5-HT plays a role in modulating the function of the HPA axis through two different mechanisms, it both regulates the feed-forward activation of the HPA axis and affects the feed-back mechanisms that regulate HPA axis activity.

4.2.2 5-HT involvement in HPA axis feed-forward drive

With regard to feed-forward regulation, Liposits et al. (1987) were the first to demonstrate that CRH containing neurones in the PVN received direct synaptic innervation from 5-HT neurones. These connections are proposed to represent functional 5-HT inputs into the PVN as 5-HT directly stimulate the release of CRH from hypothalamic explants (Calogero et al., 1989) and the destruction of 5-HT nerve terminals within the PVN, by 5,7-DHT injection, significantly attenuates the ACTH response to numerous stressors (Feldman et al., 1984).

Therefore, the ability of 5-HT precursors (tryptophan) and the releasing agent fenfluramine to stimulate ACTH release *in vivo* was proposed to result, in part, from direct activation of PVN CRH-containing neurones (Heninger et al., 1984; O'Keane and Dinan, 1991). With the development of more specific pharmacological probes it was possible to determine which 5-HT receptor subtypes were responsible for the activating effect of 5-HT on the HPA axis. Thus, a central role for 5-HT_{1A} and 5-$HT_{2A/C}$ was identified by the ability of their selective agonists, 8-OH-DPAT and DOI, to increase *in vivo* CRH and ACTH release (Lesch et al., 1990; Rittenhouse et al., 1994; Yatham and Steiner, 1993). As these studies involved systemic injection of the serotonergic agonists, however, the localisation of their action within the CNS could not be determined. Furthermore, the possible contribution of other neurotransmitter systems to their effects on the HPA axis could not be ruled out. However, as direct electrical stimulation of the dorsal raphé has been shown to excite neuronal activity within the PVN (Saphier and Feldman, 1989), and CRH neurones express 5-HT_{1A} and 5-$HT_{2A/C}$ receptors (Li et al., 1997b) the direct activation of these receptors could certainly be responsible, in part at least, for the increases in ACTH induced by systemic injection of their respective agonists (Little et al., 1998; Pan et al., 2001). Nevertheless, the contribution of other 5-HT receptor subtypes and the involvement of other 5-HT-mediated feed-forward pathways in the regulation of the HPA axis under both basal and stress conditions must not be underestimated. Indeed, the observation that 5-HT_1 and 5-HT_2 receptor antagonists, alone and in combination, can not completely inhibit the effect of endogenously released 5-HT on CRH and ACTH secretion suggests that other pathways and 5-HT receptor subtypes are of importance (Fuller and Snoddy, 1990). There is some evidence supporting a role for the 5-HT_{1B} and 5-HT_7 receptor subtypes in HPA axis activation (Jorgensen et al., 2002) and injection of the 5-HT_{2A} antagonist ketanserin directly into the amygdala attenuates the HPA axis response to photic stress (Feldman et al., 1998). These findings indicate the importance of alternate neuronal pathways to that of the direct activation of PVN CRH neurones by 5-HT, and specifically for 5-HT_{2A} receptors, in the feed-forward regulation of HPA axis activity. Evidence also suggests that 5-HT may play a role in regulating the output of ACTH by acting directly at the level of the pituitary as 5-HT, DOI and 8-OH-DPAT all elicit increased secretion of ACTH from primary rat anterior pituitary cell cultures (Calogero et al., 1993), as does 5-HT_3 and 5-HT_4 receptor activation (Calogero et al., 1995). In addition to these central mechanisms of activation, 5-HT may also act as a paracrine regulator in the adrenal cortex to stimulate corticosterone secretion via activation of 5-HT_2, 5-HT_4 (human) or 5-HT_2 and 5-HT_7 (rat) receptors on adrenocortical cells (Alper, 1990; Contesse et al., 2000; Lefebvre et al., 1992).

Despite the multitude of studies implicating a role for 5-HT in the feed-forward regulation of HPA axis functioning its true role in the stress response is yet to be fully elucidated. For example, while it has been reported that 5-HT_1 and 5-HT_2 receptor antagonists are able to attenuate the HPA axis response to specific stressors, including restraint, ether stress and immune activation (with LPS endotoxin), they do not attenuate the response to other stressful stimuli, such as cold-swim stress (Jorgensen et al., 2002; Saphier et al., 1995). This suggests that these receptor subtypes, or even the 5-HT system as a whole, is involved in modulating the effects of some stressors upon HPA axis activity but not others.

In addition to the acute regulation of HPA axis activation by altering neuronal activity and acute CRH, AVP and ACTH secretion, evidence also suggests that 5-HT may regulate the rate of CRH and ACTH synthesis through transcriptional mechanisms. 5-HT_{1A}, 5-HT_{1B} and 5-$HT_{2A/C}$ receptors have all been implicated in the control of CRH and ACTH synthesis, as administration of their selective agonists increases the expression of CRH mRNA in the PVN and that of the ACTH pre-cursor pro-opiomelanocortin (POMC) in the anterior pituitary (Jorgensen et al., 2002). Only 5-$HT_{2A/C}$ receptors, however, have been shown to regulate AVP mRNA expression (Jorgensen et al., 2003). The exact role of 5-HT in the regulation of CRH, AVP and POMC expression in response to stress, remains unclear as 5-HT depletion by p-chlorophenylalanine does not attenuate LPS stress-induced alterations in CRH mRNA expression (Condo et al., 1998; Harbuz et al., 1993). Furthermore, 5-HT depletion enhances POMC expression in the pituitary in response to restraint stress, suggesting that the 5-HT system may inhibit POMC expression during stress (Garcia-Garcia et al., 1997). An inhibitory role for 5-HT on POMC expression is further supported by the observation than chronic SSRI treatment, which increases extracellular 5-HT, mediates a down-regulation in POMC mRNA expression (Jensen et al., 1999; 2001).

4.2.3 5-HT regulation of HPA axis feed-back mechanisms

A role for 5-HT in the regulation of glucocorticoid feedback on HPA axis activity is supported by the observation that lesioning of the 5-HT system, or 5-HT depletion, reduces the ability of dexamethasone (GR agonist) to suppress corticosterone secretion (Lowy et al., 1990; Mcintyre et al., 1984). In addition, lesioning of central 5-HT pathways has been shown to decrease [^{3}H]corticosterone and [^{3}H]dexamethasone binding in CNS regions known to be involved in the negative feedback regulation of the HPA axis (Lovenberg et al., 1993; Stith

and Weingarten, 1979), and to reduce mRNA levels for both the GR and MR (Yau et al., 1994). Furthermore, studies using chronic SSRI treatment paradigms, which chronically increase extracellular 5-HT levels, have shown that glucocorticoid feedback to the HPA axis is enhanced (Pariante and Miller, 2001; Pariante et al., 2004). In human studies, this enhanced feedback can only be attributed to alterations in GR function as the ligands used to detect this (dexamethasone and prednisolone) only target the GR receptor. However, pre-clinical and *in vitro* data suggests that chronic SSRI treatment up-regulates both MR and GR function in central structures known to be involved in the negative feedback of glucocorticoids (Bjartmar et al., 2000; Okugawa et al., 1999; Pariante et al., 2001; Seckl and Fink, 1992). Despite these observations limited research has been directed towards elucidating the role of specific 5-HT receptor subtypes in the regulation of MR and GR expression although one recent study has highlighted the involvement of 5-HT_7 receptors in the regulation of hippocampal GR receptor expression (Laplante et al., 2002). Overall, the data suggests that 5-HT signalling regulates GR and MR expression as a contributory mechanism to the regulation of glucocorticoid-mediated feed-back inhibition of the HPA axis.

4.2.4 Reciprocal 5-HT system-HPA axis Interactions

In addition to widespread evidence supporting a role for 5-HT in the regulation of the HPA axis functioning a multitude of studies also suggest that the HPA axis, through both CRH and glucocortiocoid release, regulates the function of the 5-HT system. For example, increased glucocorticoid levels have been shown to increase central 5-HT turnover (DeKloet et al., 1982; Singh et al., 1990), induce a down-regulation of 5-HT_{1A} autoreceptor expression or desensitize their function (Bagdy et al., 1989; Fairchild et al., 2003a,b), increase postsynaptic 5-HT_{1A} expression in the hippocampus (Frankfurt et al., 1993; Takao et al., 1995), increase the number and sensitivity of postsynaptic 5-HT_2 receptors (Kuroda et al., 1992; Takao et al., 1995) and increases 5-HT_{1B} binding (Frankfurt et al., 1993). Additionally, 5-HT_{1A} and 5-HT_{1B} receptors, but not 5-HT_2 receptors, appear to be tonically regulated by glucocorticoid levels as adrenalectomy decreases both 5-HT_{1A} and 5-HT_{1B} receptor expression but does not alter that of the 5-HT_2 receptors (Chaouloff et al., 1995; 1993; Kuroda et al., 1992a,b). This may be related to the known regulatory role of MR but not GR signalling on 5-HT_{1A} receptor expression (Kuroda et al., 1994) and that of GR signalling on the 5-HT_2 receptors (Kuroda et al., 1993).

In addition to glucocorticoid-mediated regulation of the 5-HT system evidence also suggests that CRF may be involved in the integration between the stress-response and 5-HT system functioning. In addition to its neuroendocrine role, centrally released CRF may also act as a neuromodulator or neurotransmitter. CRF-immunoreactive terminals are widespread throughout the CNS and are present within the raphé (Sakanaka et al., 1987; Swanson et al., 1983) as are the CRF receptors (DeSouza et al., 1987). Furthermore, acute central administration of CRF reduces extracellular 5-HT concentrations in the forebrain (Price and Lucki, 2001) and CRF inhibits dorsal raphé neuronal activity when administered locally (Price et al., 1998). This suggests that CRF may represent another mechanism by which stress-related systems influence the activity of the 5-HT system.

4.2.5 Psychopathology of the HPA axis

Activity of the HPA axis is tightly regulated by complex feed-forward and feed-back mechanisms that are designed to limit the duration of HPA axis activation in times of stress. The time-limited nature of the HPA axis renders its physiological and behavioural effects, including its anti-reproductive, catabolic and immunosuppressant effects, beneficial to survival. Dysregulation of HPA axis function, however, is likely to result in a number of alterations that are damaging. Indeed, substantial evidence suggests that HPA axis dysfunction may be central in the aetiology of a number of disorders including obesity, diabetes mellitus, rheumatoid arthritis and the affective disorders where altered HPA axis function has been reported in patients suffering from major depressive disorder (Gibbons and Mchugh, 1962; Gold et al., 1986; Rubin et al., 1987), bipolar disorder (Cervantes et al., 2001; Middlemiss et al., 2002), panic disorder (Abelson and Curtis, 1996), obsessive compulsive disorder (Gehris et al., 1990; Monteleone et al., 1994), generalised anxiety disorder (Nutt, 2001) and post-traumatic stress disorder (Marshall et al., 2002).

Altered HPA axis function is most commonly reported and has been most extensively characterised in MDD patients. An increase in basal HPA axis activity, as reflected by an increase in plasma cortisol secretion throughout the 24 hour circadian cycle, is found in approximately 50% of MDD patients (Halbreich et al., 1985; Pfohl et al., 1985; Rubin et al., 1987) and evidence suggests that alterations in both the feed-forward and feed-back mechanisms regulating HPA axis function are present in depression. An increase in feed-forward drive is supported by observations of increase cerebrospinal fluid (CSF) CRF levels (Carroll et al., 1976), an increase in CRF and AVP co-localised expression in the PVN

(Raadsheer et al., 1994), a marked enhancement of the synergistic effect of AVP on CRH stimulated ACTH release (Dinan, 2004; Dinan et al., 2004) and increased plasma ACTH levels (Anisman et al., 1999). Although adrenal gland hypertrophy is commonly reported in depressed patients the sensitivity of the adrenal gland to ACTH is not altered (Amsterdam et al., 1989; Carroll, 1980; Carroll et al., 2007; Fang et al., 1988; Kalin et al., 1987; Rubin et al., 1995). The increased size of the adrenal gland in the presence of enhanced plasma ACTH secretion is likely to contribute to the increased basal plasma cortisol secretion observed in some depressed patients.

Evidence also suggests that the negative feed-back regulation of HPA axis function by glucocorticoids is attenuated in depression. Numerous studies that have reported the inability of dexamethasone, in the classic "dexamethasone suppression test" (DST), or hydrocortisone to suppress plasma ACTH and corticosterone levels in depressed patients (Carroll, 1980; Maes et al., 1991; Young et al., 1991). As dexamethasone only has agonist activity on GR receptors *in vivo* and not on the MR receptor (Reul et al., 2000) this suggests that the GR receptor system is deficient in depression (for review see (Pariante and Miller, 2001). In keeping with this suggestion, levels of GR mRNA were found to be decreased in the post-mortem brains of depressed patients (Lopez et al., 1998; Webster et al., 2002) while in contrast, evidence suggests that MR receptor function is maintained or even increased in depressed patients (Lopez et al., 1998; Webster et al., 2002). As increased 5-HT neurotransmission is associated with enhanced GR and MR expression (Pariante and Miller, 2001; Pariante et al., 2004) a deficit in 5-HT neurotransmission in depression may be central to the decreased glucocorticoid feedback found in this disorder. Therefore, altered balance in GR-MR receptor signalling is likely to contribute to the aberrant HPA axis functioning in depression.

The observation that the alterations in mood experienced by depressed patients treated with antidepressants were temporally associated with normalisation of HPA axis function has lead to the hypothesis that antidepressants regulate mood in depressed patients, in part, through their long-term effects on HPA axis function (Holsboer and Barden, 1996; Holsboer et al., 1982; Nikisch et al., 2005). In depressed patients these normalisations include a reduction in plasma ACTH and cortisol (Inder et al., 2001), a reduction in CSF levels of CRF and AVP (Debellis et al., 1993) and a reduction in adrenal size (Rubin et al., 1995).

Numerous clinical and preclinical studies have attempted to characterise the mechanisms that underlie HPA axis normalisation in depressed patients following chronic antidepressant treatment. Evidence from studies using both TCAs and SSRIs suggest that modification occurs in both the feed-forward and feed-back mechanisms regulating HPA axis activity. Evidence for reduced feed-forward drive following chronic anti-depressant treatment includes reduced CRF mRNA expression levels in the PVN (Brady et al., 1991), reduced POMC mRNA expression in the pituitary (Jensen et al., 1999) and a reduction in CRF stimulated ACTH secretion (Heuser et al., 1994) following chronic antidepressant treatment.

However, the modification of feed-back mechanisms regulating HPA axis functioning has received the most attention. A primary role for altered GR receptor function in mediating the effects of antidepressant treatment on HPA axis function was first suggested following the observation that dexamethasone suppression of plasma corticosterone was re-established in antidepressant-treated, remitted depressed patients (Greden et al., 1983; Peselow and Fieve, 1982). A multitude of preclinical studies have confirmed that long-term antidepressant treatment can upregulate both GR mRNA and binding in key regions involved with glucocorticoid feedback to the HPA axis (for review see (Pariante and Miller, 2001). Initially, it was suggested that only TCAs and not SSRIs altered HPA axis feedback by altering GR receptor levels, but several recent studies support the ability of SSRIs to modify GR receptor expression in key regulatory brain regions (Uys et al., 2006; Yau et al., 2002). In addition to the regulation of GR by anti-depressants there is also evidence for increased MR function following chronic treatment with both the TCAs and SSRIs (Pariante et al., 2004; Seckl and Fink, 1992).

4.3 Serotonin and brain development

5-HT, like the other monoamine neurotransmitters, has been shown to play a role in regulating brain development prior to the time it assumes its role as a neurotransmitter in the mature brain. The early expression of the 5-HT neurotransmitter system and its widespread distribution are proposed to be central to its known diverse effects upon brain development. 5-HT has been shown to be mitogenic for both neurones and other cell types (Lauder, 1993) and is also implicated in a multitude of other developmental events including neuronal differentiation, synaptogenesis and dendritic refinement. Through these mechanisms 5-HT influences the development and maturation of terminal areas but in addition 5-

HT has been shown to influence the development of 5-HT projections themselves, through a process known as autoregulation (Whitaker-Azmitia 2001).

In vivo pharmacological manipulations during development have shown that 5-HT depletion or 5-HT receptor activation during critical developmental periods can influence neurogenesis (Lauder et al., 1976), neuronal differentiation (Lauder et al., 1987) and neurite outgrowth (Haydon et al., 1987; Lotto et al., 1999). As SERT plays a primary role in the regulation of 5-HT availability it is likely that any manipulation which alters SERT function during these periods may also impact upon brain development. The observation that the temporal expression of SERT during brain development closely mirrors that of 5-HT (Bruning et al., 1997; Zhou et al., 2000) supports the contention that SERT may indeed regulate 5-HT availability at this time. Furthermore, the complete ablation of SERT function (in SERT knock-out mice) has been shown to result in subtle alterations in the brain proposed to be related to the role of 5-HT in development. This includes a decrease in 5-HT cell number (Lira et al., 2003) and altered barrel field formation in the somatosensory cortex (Persico et al, 2001; Salichon et al., 2001). Furthermore, as the alterations present in the barrel formation of SERT KO mice can be overcome by removal of the 5-HT_{1B} receptor (by genetic knock-out) this supports the suggestion that it is the regulation of extracellular 5-HT concentrations by SERT and the subsequent elevation of 5-HT_{1B} receptor activation, which results in altered barrel formation (Salichon et al., 2001). Pharmacological manipulation of 5-HT availability during the prenatal period has also been shown to alter the function of specific serotonin receptor subtypes in the brain of the adult animal (Whitaker-Azmitia et al., 1987). As the work presented in this thesis involves the investigation of brain and serotonin system function in an animal model with a *life-long* increase in SERT function it is important to recognise that developmental mechanisms are likely to contribute to some of the altered endophenotype found in these animals. Furthermore, the altered regulation of 5-HT in these animals during development, as a result of increased SERT function, may also contribute to the anxiolytic behavioural phenotype of these animals as pharmacological manipulation of SERT function restricted to the perinatal period

have also been shown to produce altered anxiety-like behaviour in the adult animal (Ansorge et al., 2004).

5. Animal model used in thesis

5.1 Generation of *h*SERT over-expressing (*h*SERT OVR) mice

Mice over-expressing the human serotonin transporter gene (*h*SERT OVR) were generated at the University of Edinburgh from C57BL/6xCBA wild-type (Wt) mice by Loder et al. (2000).

In brief, the transgene was a 500-Kb yeast artificial chromosome (YAC35D8) containing the hSERT gene flanked by 150kb of 5' and 300 kb 3' sequence, with the 'short' allele of the 5-HTTLPR in the promoter region and the 10-repeat allele of the VNTR in intron 2. The YAC was modified to include a hemagglutinin epitope tag at the C-terminus of the 5-HTT protein and the *lac*Z reporter gene downstream of an internal entry ribosome site using *plac*ZSERT and pYAM4 (Connor et al., 2000). The vector *plac*ZSERT was generated by:-

(i) inserting a fragment of *h*SERT genomic DNA extending 5kb upstream from the stop codon (obtained by PCR using the forward primer 5'-ACTGCATAGCGGCCGCATCTTTCATTTGCATCCC-3' and the reverse primer 5'-TGTGCTCGAGAGCATTCAAGCGGATGT-3') into the Notl-Xhol sites of pYIV3.

(ii) inserting a sequence downstream of the stop codon (obtained by PCR with the forward primer 5'-CTCCTCGAGAGGAAAAAGGCTTCT-3' and the revese primer 5'-TAGGTACCCTGTTCTCTCCTACGCAGTTT-3') into the Sall-Kpnl sites of pYIV3.

The unmodified and *plac*ZSERT-modified YAC35D8 were then transformed with Notl-linearized pYAM4 to amplify YAC DNA. YAC DNA was then purified by pulsed field gel electorphoresis and injected into the fertilised eggs of wild-type mice. Transgenic mice were identified by PCR using primer pairs for *h*SERT (exon 1A, 1B, inton 1A and 3'UTR), STS markers (D17S2009, D17S2004, D1S1294 and D17S1549) and the YAC vector arms (Shen et al., 2000a).

Mice from the A102.3 transgenic line previously partially characterised (Loder et al., 2000; Jennings et al., 2006; MacLean et al., 2004) were used in this study. This transgenic line was chosen as it was considered likely to contain the complete *h*SERT transgene, expressing all *h*SERT gene coding regions and the STS markers, which are located close to the *h*SERT gene. The A102.3 transgenic line was found to have 8 copies of the transgene, which resulted in a pronounced increase in SERT mRNA (approximately 3-fold) and total SERT protein expression levels (approximately 3-fold) in the central nervouse system (Jennings et al., 2006; Loder et al., 2000; 2002).

In addition to the human SERT gene (SLC6A4) the transgene present in *h*SERT OVR mice also contains genes coding for a number of other proteins, including that for bleomycin hydrolase, the carboxypeptidase D precursor and the golgi SNAP receptor complex member 1. While the inclusion of these genes in the transgene presents an important limitation when considering the possible influence of the transgene on parameters of serotonergic system functioning, at present there is no evidence to suggest that any gene present in the transgene other than that for the *h*SERT is involved in the regulation of 5-HT system functioning.

5.2 Serotonergic characteristics of *h*SERT OVR mice

An increase in SERT mRNA expression has been confirmed in the CNS of *h*SERT OVR mice from the A102.3 transgenic line by in situ hybridisation. Furthermore, an increase in SERT binding site density (2.3-3.5 fold) and an alteration in the Hill co-efficient, indicative of the presence of more than 1 binding site, has also been reported in cortical membranes of *h*SERT OVR as compared to wild-type (Wt) mice (Jennings et al., 2006). This not only confirms that SERT expression is increased in the CNS of mice with the *h*SERT transgene but also suggests that these mice express both the murine and human form of the transporter, as the rodent and human SERT have differing affinities for the SERT ligands (MacLean et al., 2004; Plenge and Mellerup, 1991). The increased SERT expression in the CNS of *h*SERT OVR mice results in decreased extracellular and tissue 5-HT levels while tissue 5-HIAA levels are not affected (Jennings et al., 2006).

5.3 Relevance of *h*SERT OVR mice to Humans

As previously outlined (section 4.6.3) polymorphic variations in the human SERT gene exist that lead to a life-long increase in SERT function. For example, the “long”/“L” promoter of

the 5-HTTLPR region results in greater transcriptional activity of the hSERT gene in comparison to the "short"/"S" promoter and results in higher SERT mRNA expression, protein density and 5-HT uptake in lymphoblastoid cells, platelets and the brain (Greenberg et al., 1999; Heinz et al., 2000; Lesch et al., 1996; Little et al., 1998). Several studies have investigated the possible relationship between the 5-HTTLPR and affective psychopathology. However, despite the known influence of the 5-HTTLPR on affective functioning, and involvement in the risk of developing affective disorders (as reviewed below), the underlying differences in neurobiology and physiology which contribute to these effects are unknown. Research into these parameters has been limited by the fact that the 5-HTTLPR sequence is present in humans and non-human primates but not in rodents (Lesch et al., 1996) and that the function of known transcriptional control elements in murine SERT are not yet sufficiently well characterised to allow experimental alterations that would drive increased SERT expression. However, *h*SERT OVR mice have a life-long increase in SERT function which parallels that observed in "L" allele human individuals as compared to "S" allele individuals. These animals, therefore, provide a unique opportunity in which to investigate the mechanisms by which a life-long increase in SERT expression may regulate affective functioning and the underlying neurobiology. Here, I briefly review evidence from clinical studies implicating a role for the 5-HTTLPR in psychopathology.

5.3.1 5-HTTLPR polymorphism and Psychopathology

A role for the 5-HTTLPR in the risk of developing and influencing the severity of several psychopathologies has been reported. For example, the "S" allele has been associated with increased impulsivity and aggression (Beitchman et al., 2006; Sakado et al., 2003; Steiger et al., 2005), is more frequent in anorexia and bulimia patients (Di Bella et al., 2000; Fumeron et al., 2001; Matsushita et al., 2004), and is also more frequent in migraine sufferers (Gonda et al., 2007; Juhasz et al., 2003) as compared to controls. However, an equal number of studies have reported no association, or no difference in the frequency of the "S" allele as compared to controls in patients with the same disorders (Baca-Garcia et al., 2004; Patkar et al., 2002; Schmidt et al., 2002; Hinney et al., 1997; Sundamurthy et al., 2000; Urwin et al., 2003; Yilmaz et al., 2001). At present meta-analysis studies have not been completed to further investigate the possible relationship between any of these disorders and the 5-HTTLPR polymorphism.

The usefulness of meta-analysis studies in considering the effect of the 5-HTTLPR polymorphism in psychopathology can be demonstrated by studies investigating the effect of the polymorphisms on suicidal behaviour. A possible role for the 5-HTTLPR in suicide is supported by several studies which have reported an increased frequency of the "S" allele in hospitalised violent suicide attempters (Bayle et al., 2003; Bellivier et al., 2000) and suicide completers (Bondy et al., 2000). An association between the "S" allele has also been reported with a family history of suicide (Joiner et al., 2002; Limosin et al., 2005) and a personal history of suicide attempts (Limosin et al., 2005; Preuss et al., 2001). However, numerous others have reported no such association (Courtet et al., 2003; Mann et al., 2000; Rujescu et al., 2001; Segal et al., 2006). Furthermore, other studies have demonstrated a significantly higher frequency of the "L" allele in depressed suicide victims in comparison to non-suicidal controls (Du et al., 1998; 1999). To clarify the relationship between the 5-HTTLPR and suicide a recent meta-analysis of 25 studies investigating the relationship between the 5-HTTLPR and suicide was completed. This has shown that although the "S" allele does not increase the risk of suicide, when comparing suicidal individuals to normal controls, it did significantly increase the risk of suicide attempts in psychiatric patients with the same psychiatric diagnosis and was also associated with an increase in the level of violence in the method used to commit suicide (Lin and Tsai, 2004).

Where the "S" allele may confer increased susceptibility to some disorders it is apparent that the "L" allele may play a role in determining either the likelihood or clinical severity of other disorders. For example, while the 5-HTTLPR polymorphism was not found, by meta-analysis, to influence susceptibility to the development of schizophrenia (Fan and Sklar, 2005) a number of studies have associated the "L" allele with an increase in the severity of the schizophrenic hallucinations and the schizo-affective symptoms experienced by patients (Kaiser et al., 2001; Malhotra et al., 1998). Furthermore, the "L" allele has been reported to be more frequent in autism (Yirmiya et al., 2001) and has been associated with an increase in the severity of social and communicative impairments in autistic individuals (Tordjman et al., 2001). In addition, the "L" allele has also been reported to be more frequent in patients suffering from OCD (Greenberg et al., 1999).

5.3.2 5-HTTLPR and Anxiety

Since the seminal study by Lesch et al (1996) detailing the influence of the 5-HTTLPR on anxiety-related traits (neuroticism scores on the NEO-PI-R) many subsequent studies have

replicated the finding that the "S" allele of the 5-HTTLPR is associated with increased neuroticism (Greenberg et al., 2000; Osher et al., 2000; Schinka et al., 2004). This relationship has also been confirmed in a number of other studies using different measures of anxiety-related personality traits, including the harm avoidance scale (of the Tridimensional Personality Questionaire (TPQ)) (Heinz et al., 2000; Osher et al., 2000; Tsai et al., 2002) and self-rating anxiety and depression questionnaires (Murakami et al., 1999). However, several studies have reported no association between the 5-HTTLPR and anxiety traits with the same measures (Ebstein et al., 1997; Gustavsson et al., 1999; Lerman et al., 2000; Willis-Owen et al., 2005). These inconsistencies may be influenced by the small sample sizes used in some studies and the possible interference of other co-morbid disorders in some patient samples. However, a recent meta-analysis of 26 studies investigating the possible influence of the 5-HTTPLR polymorphism on anxiety traits suggests that the "S" allele does indeed increase neuroticism (as detected by NEO) but not harm avoidance or other anxiety-related personality traits (Mintun et al., 2004).

Despite the significant influence of the 5-HTTLPR on neuroticism, however, few investigations have found evidence for an increase in the frequency of the "S" allele in patients with anxiety disorders (You et al., 2005). Indeed, most studies fail to demonstrate any significant difference in the frequency of the "S" allele in patients with these anxiety disorders (Deckert et al., 1997; Matsushita et al., 1997; Olesen et al., 2005). However within these patients a link between the "S" allele and an increase in the severity of some of the symptoms of these disorders has often been noted.

5.3.3 5-HTTLPR and Depressive Disorders

As neuroticism is a governing factor in the likelihood of developing depression (Collier et al., 1996; Duggan et al., 1995) and neuroticism is influenced by the 5-HTTLPR, research has been directed towards investigating the possible influence of the 5-HTTLPR in depressive disorders. Multiple studies provide evidence for an association between the "S" allele and the risk of developing depression, with an increased frequency of this allele identified in patients with MDD (Furlong et al., 1998; Hoefgen et al., 2005; Heils et al., 1997; Mann et al., 2000), BPD (Furlong et al., 1998; Rees et al., 1997) and seasonal affective disorder (Rosenthal et al., 1998). However, several other studies have found no evidence for a significant difference in the frequency of the "S" promoter in depressed patients (Gutierrez et al., 1998; Steffens et al., 2002) and no association between the 5HTTLPR polymorphism and

MDD (Frisch et al., 1999; Gillespie et al., 2005; Hoehe et al., 1998), BPD (Kirov et al., 1999; Mellerup et al., 2001) or seasonal affective disorder (Johansson et al., 1997). Possible reasons for variability in the findings of these studies include the use of small sample sizes, variability in the clinical phenotypes of subjects included in the studies and possible interference from coexisting disorders. The relative distribution of male and female participants in the sample may also be important (see section 7.2). Despite these contrasting results, however, two meta-analysis have confirmed that individuals homozygous for the "S" allele (S/S genotype) are at increased risk of suffering from MDD and BPD (Furlong et al., 1998; Lotrich and Pollock, 2004).

Recent research suggests that the effect of the 5-HTTLPR polymorphism on the likelihood to develop depression is directly mediated by its effect on neuroticism (David et al., 2005) while others have suggested that the modulatory effect of the 5-HTTLPR on the response to life stressors may be central to its influence on the likelihood of developing depression (Caspi et al., 2003). However, the close inter-relationship between neuroticism and the perception of stress makes it difficult to delineate each of these factors as the primary mechanism by which the 5-HTTLPR influences the vulnerability to depression, and it is the interaction between these factors which is likely to be most important.

5.3.4 5-HTTLPR and Antidepressant Efficacy

In addition to its influence on the vulnerability to developing affective disorders the 5-HTTLPR polymorphism has also been found to influence the efficacy of antidepressant treatment in these disorders. A number of studies have reported decreased efficacy of antidepressant treatment in patients with an "S" allele (S/S or S/L genotype) (Arias et al., 2003; Pollock et al., 2000; Zanardi et al., 2001) while others have reported decreased efficacy in patients homozygous for the "S" allele (S/S genotype) (Smeraldi et al., 1998). However, the association between the 5-HTTLPR and the efficacy of antidepressant treatment are not always consistent (Smits et al., 2004). The considerations previously noted with regard to studies investigating the frequency of the 5-HTTLPR in affective disorders are also relevant to these studies. In addition ethnicity may be an important factor in the variability observed in these studies. Thus, a number of studies conducted in Asian populations have reported that antidepressant efficacy is increased in "S" allele as compared to "L" allele patients (Kim et al., 2000; Kim et al., 2006; Yoshida et al., 2002). This is in direct contrast to the effect reported by studies in Caucasian populations (Arias et al., 2003;

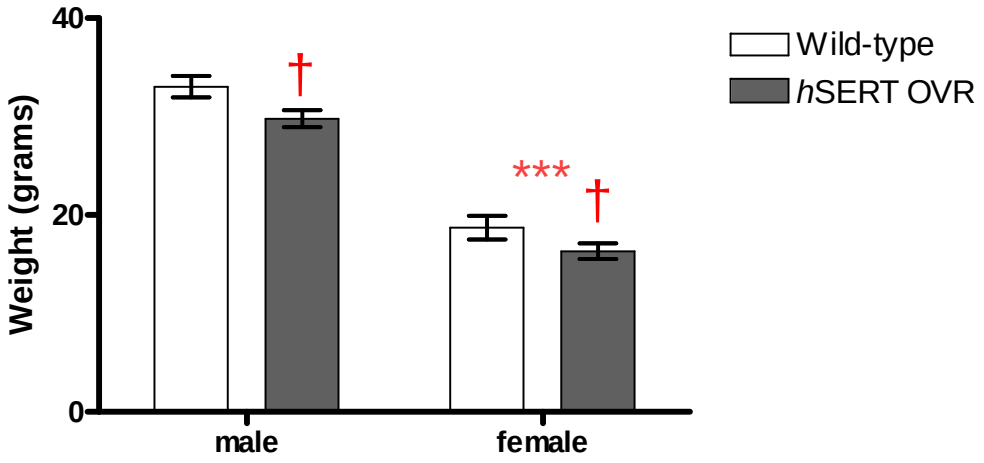
Durham et al., 2004; Joyce, 2003; Serretti et al., 2007) and suggests that ethnicity may modulate the ability of the 5-HTTLPR polymorphisms to influence the antidepressant response. However, the possible influence of ethnicity requires further investigation as a number of more recent studies in Asian populations have reported the same trend as that observed in Caucasian samples (Cheetham et al., 1988; Hong et al., 2006). Furthermore, a recent meta-analysis of 15 studies investigating the possible effect of the 5-HTTLPR polymorphism on SSRI efficacy in depressed patients, which included studies with both Asian and Caucasian samples, found that being homozygous for the "S" allele significantly reduced the frequency of remission from depressive symptoms, whereas being both homozygous or heterozygous for the "S" allele significantly delayed the rate of the treatment response (Serretti et al., 2007).

Overall, the data from human studies suggest that the "S" allele of the 5-HTTLPR may be associated with an increased risk of individuals developing affective disorders, in part through its influence on trait anxiety, and that this allele is also associated with the reduced efficacy of antidepressant treatment.

5.4 Phenotype of *h*SERT OVR mice

In comparison to Wt animals *h*SERT OVR mice display decreased anxiety-related behaviour in two behavioural paradigms, the elevated-plus maze (EPM) and the hyponeophagia test (Jennings et al., 2006). This suggests that *h*SERT OVR mice may provide a valid model of the decreased trait anxiety observed in humans with the "L" 5-HTTLPR polymorphism. During these studies we also noted that *h*SERT OVR mice displayed a decreased body weight in comparison to age-matched Wt mice (Figure 1.7).

Figure 1.7 Effect of *h*SERT OVR on Body Weight



*Body weight of age-matched male and female Wt and hSERT OVR mice. Data from mice used in basal [^{14}C]2-deoxyglucose experiment (saline-treated). ***denotes p<0.001 significant gender difference, †denotes p<0.05 significant genotype effect (2-way ANOVA). hSERT over-expression results in a significant decrease in body weight in both male and female animals.*

6. Importance of Gender in Affective Disorders

Gender represents a significant factor in susceptibility for the development of both depressive and anxiety disorders with women approximately twice as likely to develop these disorders than men (Kessler et al., 1994) . Clinical evidence also suggests that the severity of symptoms is greater and the onset of effective antidepressant treatment delayed in depressed women as compared to men (Drevets et al., 2002; Kornstein et al., 2000; Kornstein et al., 1995). Furthermore, preliminary data also suggest that treatment of GAD with SSRI antidepressants may be less effective in women as compared to men (Simon et al., 2006).

The neurobiology and physiology underlying the gender differences in affective disorders is largely unknown. However, reported gender differences in 5-HT and HPA axis functioning are likely to play a role. In humans gender differences reported in the 5-HT system include increased SERT (Arato et al., 1991; Staley et al., 2006), increased 5-HT$_{1A}$ (Arango et al., 1995; Parsey et al., 2002) and decreased 5-HT$_2$ receptor binding (Biver et al., 1996) along with a reduced rate of 5-HT synthesis (Nishizawa et al., 1997) in women as compared to men. Several of these sexually dimorphic differences have also been reported in studies

using experimental animals (Frankfurt et al., 1993; Little et al., 1998; Rubinow et al., 1998; Schiller et al., 2006). Despite these observations, however, there is a relative paucity of data on gender differences in 5-HT system function, especially with regard to the other 5-HT receptor subtypes and actual receptor functioning in comparison to known receptor binding differences. Nevertheless, a central role for the modulation of 5-HT system functioning by the gonadal steroids (estrogen and testosterone) is supported by a body of evidence reporting on the effects of ovariectomy, castration and hormonal supplementation (for review see (Rubinow et al., 1998) on the 5-HT system, highlighting one important mechanisms contributing to the gender differences present in the 5-HT system. In addition to the regulation of 5-HT system function by gonadal steroids, however, it is also important to note that steroids are likely to have an important organisational effect on the 5-HT system during development. Furthermore, there is increasing evidence for primary genetic mechanisms governing sexual differentiation (Reisert and Pilgrim, 1991) which are likely to influence the 5-HT system.

Considering the suggested central role of the HPA axis in the aetiology of depressive and anxiety disorders it is of interest that gender differences in HPA axis function have also been reported. In the preclinical literature gender differences in HPA axis functioning are consistent, with female reported as displaying higher basal corticosterone levels as compare to males (Chisari et al., 1995; Griffin and Whitacre, 1991; Kitay, 1961) and greater HPA axis activation in response to a number of different stressors (Lesniewska et al., 1990; Rivier et al., 1999). These observations are paralleled by evidence for increased feed-forward drive, including increased hypothalamic CRH mRNA (Patchev et al., 1995), AVP mRNA (Viau and Meaney, 1991) and increased plasma ACTH levels (Lemevel et al., 1979) in females as compared to males. Gender differences in HPA axis activity are influenced, in part, by the differences in gonadal steroids between the sexes with a stimulatory role for oestrogen and an inhibitory role for testosterone on HPA axis function (Seale et al., 2004). In addition to gender differences in the feed-forward mechanisms of the HPA axis decreased GR and MR receptor levels in the CNS of females as compared to males also suggest that the negative feed-back regulation of HPA axis activity may be lower in females and the gonadal steroids have also been implicated directly in the control of GR and MR function (Turner et al., 1990; 1997). In addition to the observed differences in HPA axis function between males and females evidence suggest that the programming of HPA axis activity, into a secretory pattern similar to that observed in the affective disorders, by adverse early-life experiences is more sensitive in females than in males (Matthews, 2002; Mccormick et al., 1995; Weinstock et

al., 1992). This further supports a role for the HPA axis in determining the gender differences in the likelihood of developing affective disorders.

In contrast to preclinical studies evidence from psychosocial stress studies in healthy human individuals support enhanced HPA activation by psychosocial stress in males as compared to females (Kirschbaum et al., 1999; Kirschbaum et al., 1992; Stoney et al., 1987). Furthermore, under basal conditions increased ACTH secretion in men as compared to women has also been reported although this is not accompanied by differences in cortisol levels (Horrocks et al., 1990; Roelfsema et al., 1993). These observations seem to be in direct contradiction to the hypothesis made from preclinical studies that enhanced HPA axis activity in females may play a part in their enhanced risk of developing affective disorders. However, more recent studies completed in humans using different kinds of stressors have shown that while HPA axis activity is greater in males than females during stressful challenges based on achieving a goal, females experience a greater degree of HPA axis activation in stressful challenges involving social rejection (Stroud et al., 2002). Clearly, further research needs to be dedicated to elucidating the gender dependent effects of different stressor on HPA axis activity and to understanding how these relate to the real life situations experienced by men and women.

6.1 Gender modulation of 5-HTTLPRs influence on affective functioning

Despite the known influence of gender in affective disorders and on trait anxiety the possible interaction between gender and the influence of the 5-HTTLPR on affective functioning is not clear. Several studies indicate that an interaction between these factors may exist, although the results from these studies are often contradictory. For example, while Lesch et al. (1996) and Greenberg et al. (2000) have shown that the “S” allele is associated with increased neuroticism in both male and female samples others have reported that the “S” allele only increases neuroticism in males and not in females (Brummett et al., 2003; Du et al., 2000). Furthermore, it has also been reported that the “S” allele has a diametric influence on trait anxiety in males (increased) and females (decreased) (Gelernter et al., 1998; Mizuno et al., 2006). In addition the “S” allele has been reported to increase the risk of depressive symptoms in young females but decrease them in young males (Sjoberg et al., 2006). Despite reports supporting an interaction between gender and the influence of the 5-HTTLPR on affective functioning numerous other studies have reported no such interaction (Ball et al., 1997; Ricketts et al., 1998). Furthermore, two recent meta-analyses investigating the possible

influence of gender on the association between the 5-HTTLPR and neuroticism have found no significant evidence for this (David et al., 2005; Mintun et al., 2004). Gender has also been found not to influence the modulatory effect of the 5-HTTLPR on amygdala function in response to fearful stimuli (Harari et al., 2002). The reasons for the variability in the results of these studies are largely unknown but possible confounding factors include the different ethnic backgrounds and ages of participants within the study and the use of small sample sizes, which results in low statistical power. Investigating the potential role of gender in modulating the effect of a life-long increase in SERT functioning, as affected by the 5-HTTLPR, on affective and 5-HT system functioning warrants further systematic investigation.

7. Aims of Thesis

The studies conducted in this thesis aim to elucidate the alterations in brain function that occur as a result of a genetically determined life-long increase in the expression and function of SERT, with particular emphasis on the serotonin system and HPA axis function. In these studies mice over-expressing the *h*SERT gene were used as a model of the life-long increase in SERT function present in humans with the "L" allele of the 5-HTTLPR as compared to those with the "S" allele, which were modelled by Wt animals. Alterations in constitutive brain functioning in *h*SERT OVR mice were first assessed by observing local cerebral glucose utilisation (LCMRglu) under basal conditions. As the measurement of LCMRglu in this thesis involved the use of a novel approach the validity of this method was assessed prior to its use.

Possible alterations in serotonin receptor subtype (5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A} and 5-HT_{2C}) pharmacology and function were also determined in *h*SERT OVR mice, not only to characterise the influence of a life-long increase of SERT functioning on these receptors but to further elucidate their possible involvement in the altered constitutive brain functioning observed in *h*SERT OVR mice. Constitutive brain function and serotonin receptor functioning were characterised in both male and female animals to further investigate the influence of gender on brain and serotonin system function and to further investigate the possible modulatory influence of gender on the effects of *h*SERT over-expression on these parameters.

The functional impact of a life-long increase in SERT on HPA axis functioning was also addressed by determining levels of circulating ACTH and glucocorticoids under basal conditions and in response to acute stress. Possible alterations in serotonergic feed-forward drive of the HPA axis in *h*SERT OVR mice were assessed by characterising these hormonal responses to specific 5-HT receptor agonists. Central MR and GR mRNA expression levels were also determined in *h*SERT OVR mice in order to investigate the possible influence of a life-long increase in SERT functioning on the feed-back regulation of HPA axis functioning.

Chapter 2- Methods

1. Animal Housing

All experiments were performed on male and female *h*SERT OVR mice and their Wt littermates aged 1.5-4 months (males: 24g-38g; females 16g-28g). Animals were group-housed (5-6 animals/cage) under strict environmental conditions with a 12-hour light-dark cycle (lights on 7.00-19.00) and at a temperature of 21±1^{o}C. Access to food and water was *ad libitum* until the day of the experiment. Access to food was restricted over-night (13-14 hours prior to experimental manipulation) for animals involved in 2-deoxyglucose imaging experiments to reduce the known hyperglycaemic response of specific pharmacological challenges.

2.Polymerase Chain Reaction (PCR)

2.1 Theory

The polymerase chain reaction (PCR) is an *in vitro* technique that allows for the generation of multiple copies of a target DNA sequence by repeatedly cycling through three reaction steps: DNA denaturation, primer hybridization and DNA polymerase extension. In the first step double stranded DNA is denatured into two single strands at high temperature (95^{o}C-97^{o}C). This subsequently allows other complementary DNA sequences to anneal to it by hydrogen bonding in a nucleotide specific manner. In the second step the temperature is reduced (approximately 55^{o}C-72^{o}C) to allow primer hybridization with specific sequences within the target DNA. Primers are single strands of oligonucleotides, approximately 20 nucleotides long, which complement a sequence of nucleotides flanking the target DNA. Following primer hybridisation a complementary sequence of nucleotides to those present in the target DNA is generated through the extension of each annealed primer by DNA polymerase from deoxyribonucleotide trisphosphates (dNTPs) present in the reaction mix. In PCR a thermostable polymerase originally isolated from the thermophilus aquaticus bacterium, known as Taq polymerase, is used for this extension reaction (optimum temperature 72^{o}C). Following the formation of this new single strand of DNA the sequence is repeated. Importantly, all previously synthesised DNA products act as templates for new primer-extension reactions in each new cycle, thus the DNA product is synthesised in an exponential fashion. After approximately 30 PCR cycles enough (~2^{30}) copies of the target

DNA sequence are present for it to be visualised, usually by ethidium bromide staining, following isolation of the target DNA sequence according to its mass by gel electrophoresis.

2.2 Detection of the *h*SERT transgene

Genomic DNA was extracted from ear punch tissue samples by digestion with proteinase K (600µg/ml) in a lysis buffer (50mM Tris-HCl pH8, 100mM EDTA, 100mM NaCl, 1% SDS) overnight at 56^{o}C (500µl per sample). Following digestion samples were centrifuged (13,000 rpm for 5 minutes) to remove debris and isolate DNA within the supernatant. The DNA was then precipitated from the supernatant in a fresh eppendorf tube containing 300µl of isopropanol for 15 minutes at room temperature and the DNA pellet isolated by centrifugation (13,000 rpm for 10 minutes). Excess isopropranol was then removed from the tube and the DNA pellet washed with 70% ethanol (pulse centrifugation) and re-suspended in 100µl of TE (10mM Tris-HCl, 1mM EDTA) solution by incubation for 1 hour at 56^{o}C.

1µl of each DNA sample was added to 24µl of the PCR reaction mixture (table 2.1) containing primers specific for the *h*SERT gene (MWG Biotech AG) and was then overlayed with a drop of mineral oil. A blank sample was also generated by addition of 1µl deionised water to 24µl of PCR solution. Samples then underwent PCR as outlined in table 2.2. Following PCR 5µl blue loading gel was added to each sample and the DNA samples were separated by electrophoresis (125V for approximately 1 hour) on a 2% agarose gel (with 12.5ml/112.5ml Tris-Borate-EDTA (TBE, 10x concentrate, Sigma U.K) and 5µl 10mg/ml ethidium bromide) in a 1:10 dilution TBE buffer along with a 100 base pair DNA ladder (10µl with 5µl blue loading gel, Promega, U.K). Presence of the *h*SERT gene in transgenic *h*SERT OVR mice was confirmed by observing a single 230 base pair DNA band while this product is not present in wild-type animals (Figure 2.2).

Figure 2.1 Primer Sequences

Forward Primer Sequence: 5′-TTC TTT CCT TAC TAA GTT GAG AAC G-3’

Reversed Primer Sequence: 5′-TGG CAT GCA ATT GTA GTC TC-3′

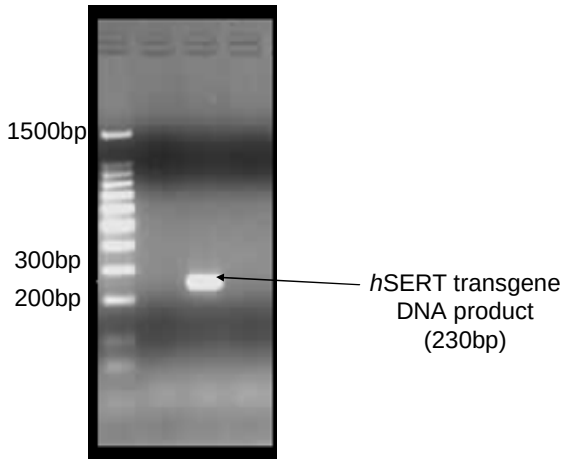
Table 2.1 PCR reaction mixture

	Volume (per sample)
Buffer (10x concentrate)	2.5µl
Magnesium Chloride	1.5µl
dNTP mix (40mM)	0.5µl
Forward Primer (200ng/µl)	0.25µl
Reverse Primer (200ng/µl)	0.25µl
TAQ polymerase	0.25 µl
ddH_2O	18.75µl
Total PCR tube volume	**24µl**

Table 2.2 PCR cycle temperature and duration for *h*SERT detection

Denature	97°C	2 minutes
5 cycles	94 °C	30 seconds
	58 °C	30 seconds
	72 °C	60 seconds
30 cycles	94 °C	30 seconds
	56 °C	30 seconds
	72 °C	60 seconds
final extension	72 °C	10 minutes

Figure 2.2 *h*SERT gene product detection on 2% agarose gel

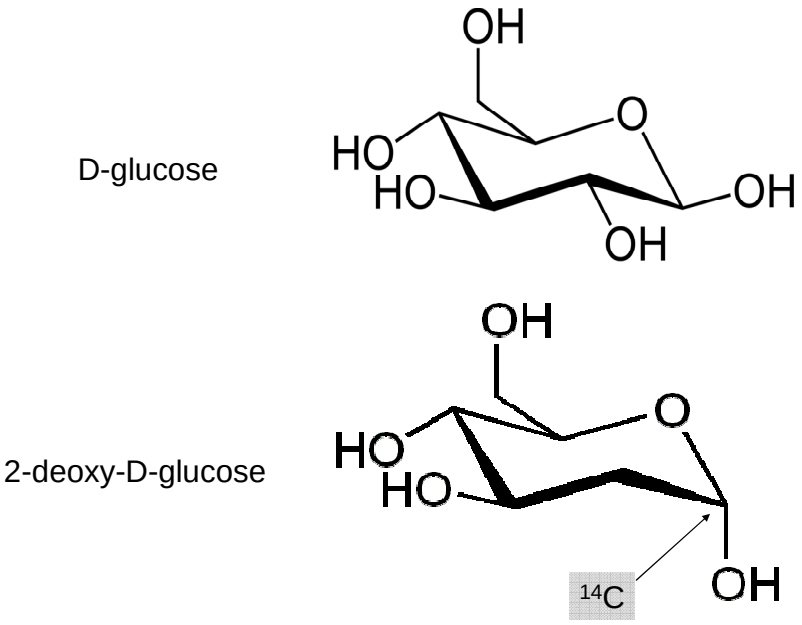


Detection of the hSERT gene product (203bp) on 2%agarose gel. First well shows the 100bp DNA ladder while the second well shows the result from a blank control (no product detected). The third well shows the result given from a hSERT OVR mouse (230bp DNA product detected) where the fourth shows the result from a Wt mouse (no 230bp product).

3. Measurement of Local Cerebral Glucose Utilisation (LCMRglu)

Under normal physiological conditions the energy requirements of the brain are provided almost exclusively by the oxidative catabolism of glucose. Importantly, the functional activity within any regions of the CNS is directly linked and intimately related to the energy consumption, and so glucose catabolism, within that region. As very low levels of glucose are stored within the CNS any functionally-related glucose requirements must be met by glucose from the cerebral blood. Use of radiolabelled glucose (or oxygen) in autoradiographic methods would not give an accurate reflection of the rate of metabolism within a brain region, as during metabolism the isotopes present in these tracers would become incorporated into carbon-dioxide (CO_2) or water (H_2O) which freely diffuse away from the brain region of interest (ROI). However, quantification of glucose utilisation as a reflection of functional activity in distinct brain regions has been made possible by the development of the [^{14}C]-2-deoxyglucose technique (Sokoloff et al., 1977).

Figure 2.3 Structure of D-glucose and 2-deoxy-D-glucose



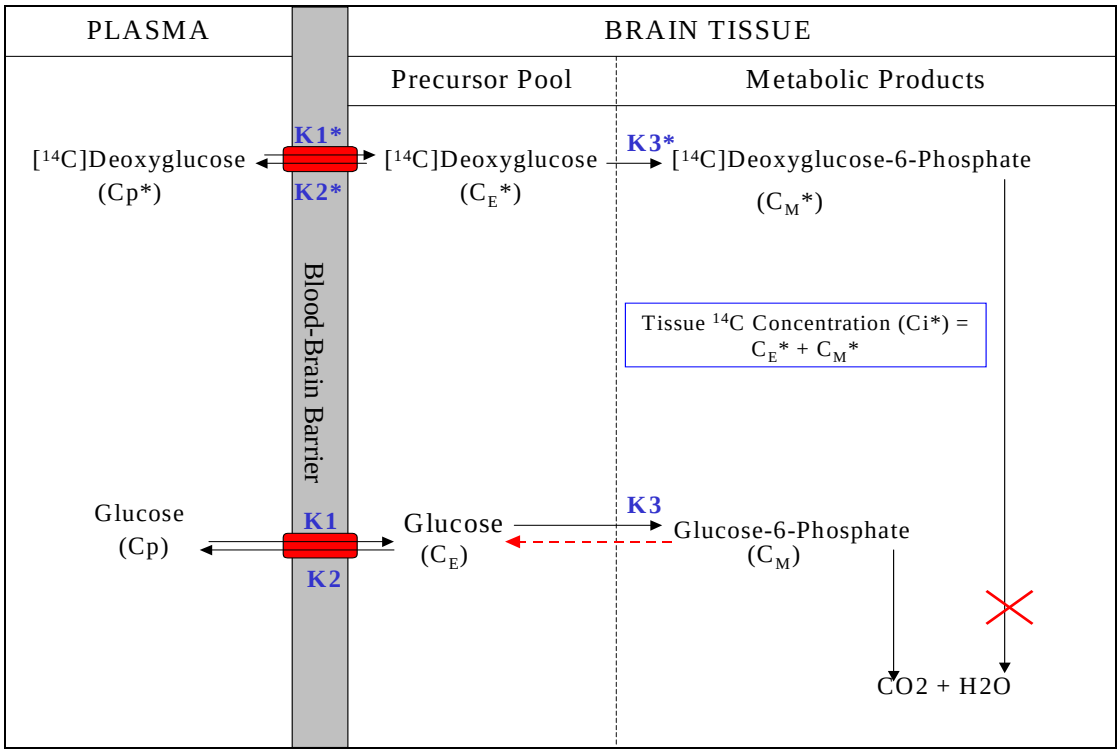
2-deoxy-D-glucose (2-DG) is an analogue of glucose with one structural difference, the replacement of the hydroxyl group on the second carbon of the hexose ring by a single hydrogen atom (Figure 2.3). The structural similarities between these molecules mean that they are both transported across the blood brain barrier (BBB) by the same molecular carrier and are metabolised to their respective hexose-6-phosphates by the enzyme hexokinase in

brain tissue. However, while glucose-6-phosphate can continue through the glycolytic pathway and tricarboxylic acid (TCA) cycle, being subsequently metabolised into CO_2 and H_2O, deoxyglucose-6-phophate (2-DG-P) can not and remains effectively trapped in the tissue. These biochemical characteristics of deoxyglucose mean that radiolabelled dexoyglucose may be used in experimental animals to determine tracer accumulation over a known time period in a ROI as an accurate reflection of glucose metabolisms and so functional activity.

3.1 Mathematical model and Operational Equation for the Quantitative 2-deoxyglucose technique

Sokoloff et al. (1977) developed a mathematical model for the accumulation of radiolabelled [^{14}C]-2-DG in brain tissue that allows for the determination of local cerebral glucose utilisation (LCMRglu) in discrete regions of the CNS. This model includes pre-determined rate constants for the transport and phosphorylation (by hexokinase) of glucose and deoxyglucose (Figure 2.4). From this model and its empirically derived rate constants the "operational equation" for the 2-DG method was developed that allows for the quantification of LCMRglu.

Figure 2.4 Theoretical model of 2-deoxyglucose technique

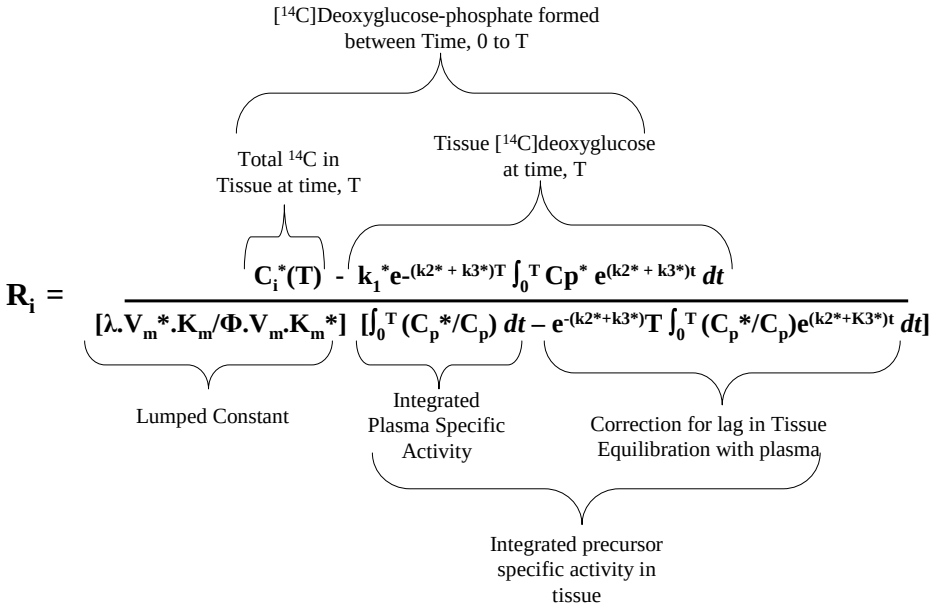


Schematic representation of the theoretical model on which the 2-deoxyglucose technique is based. Ci represents the total ^{14}C present in a single homogenous brain tissue. Cp* and Cp represent the concentrations of [^{14}C]-2-DG and glucose present in the arterial plasma, respectively. CE* and CE represent their respective concentrations in the pool available for hexokinase metabolism. CM* represents the concentration of [^{14}C]-2-DG-P present in the tissue. K1*, K2* and K3* represent the constants for the carrier-mediated transport of [^{14}C]-2-DG from plasma to tissue, tissue to plasma and hexokinase phosphorylation respectively, whereas K1, K2 and K3 represent those of glucose for the same processes. The dashed arrow represents the possibility of glucose-6-phosphate metabolism by glucose-6-phosphatase activity, if any. Adapted from Sokoloff et al., 1977.*

The operational equation outlines the determination of cerebral glucose use (Ri) following a bolus intravenous injection of [^{14}C]-2-DG when the concentrations of arterial plasma [^{14}C]-2-DG (Cp*) and glucose (Cp), gained from blood samples taken throughout the experimental period, and the total [^{14}C] concentration in a brain region, as determined by autoradiography, are known. As the concentration of [^{14}C] within a brain region reflects both the phosphorylated and unphosphorylated [^{14}C]-2-DG present in the tissue, the operational equation includes an integral determined from the arterial plasma [^{14}C] values along with the rate constants for the transportation of DG between tissue and plasma (k1* and k2*) and the phosphorylation of DG (k3*) that allows for calculation of the unphosphorylated DG present. This allows for the determination of the amount of phosphorylated DG present in the

designated brain region which relates directly to the rate of glucose utilisation. The equation also contains a "lumped constant" combining six different constants that describe the differences in the kinetics and distribution volumes of glucose and [^{14}C]-2-DG. The lumped constant and rate constants used in the operational equation have all been previously determined by Sokoloff et al. (1977) in separate experiments. In the equation the lumped constant is multiplied by the plasma integral for glucose and deoxyglucose determined throughout the experiment, and is also corrected for the lag in tissue-plasma equilibration.

Figure 2.5 Operational equation of the quantitative 2-deoxyglucose method



For abbreviations see those in legend of figure 2.4. In addition, T represents the time at which the experiment is terminated (~45 minutes). In the lumped constant λ represents the ratio of the distribution space between [^{14}C]-2-DG to glucose in the tissue, while Φ represents the fraction of glucose which continues along the glycolytic pathway. Vm, Km* and Vm, Km represent the Michaelis-Menten kinetic constants of hexokinase for [^{14}C]-2-DG and glucose, respectively.*

In order for the operational equation to give a valid measurement of LCMRglu several conditions must be met. Measurements must only be made in localised brain regions in which blood flow and the rate of uptake and phosphorylation of 2-DG are constant. The concentration of 2-DG and glucose within each element of tissue must be constant, they must be present in the same compartment and they must also be free to exchange between the

plasma and tissue. In addition, the determined arterial plasma glucose and 2-DG concentrations must accurately represent their cerebral capillary concentrations. Finally, [^{14}C]-2-DG and [^{14}C]-2-DG-P must only be present in pharmacologically inactive, tracer amounts.

It has previously been acknowledged that there is considerable imprecision in our knowledge of the rate constants (k1*, k2* and k3*) within a single animal and that the assumption in the operational equation that these constants do not change over a given range of physiological conditions is erroneous (Orzi et al., 1988). However, the error inherent in the rate constants and their influence on Ri can be diminished by used of an long (45 minute) experimental time period. As plasma [^{14}C]-2-DG concentration (Cp*) diminishes with time the use of a 45 minute experimental end-point minimises the contribution of the rate constants to the equation, therefore any error as a result of the rate constants is reduced. This has been demonstrated experimentally (Sokoloff et al., 1977; Sokoloff, 1978).

As the lumped constant represents a significant contribution to the operational equation any alteration in this constant has the potential to induce error. Theoretically, the value of the lumped constant may be altered by any factor producing an imbalance between glucose supply and utilisation. However, the lumped constant has been shown to remain stable during changes in glucose utilisation, cerebral blood flow and during moderate hypoglycaemia and hyperglycaemia. However, out-with normoglycaemia the ratio of distribution between glucose and [^{14}C]-2-DG may be altered resulting in the inaccurate calculation of LCMRglu. For example, in severe hyperglycaemia (plasma glucose in excess of 17µmol/ml) decreases in the brain distribution space of [^{14}C]-2-DG and glucose occur, leading to a decrease in the ratio of the distribution volumes for glucose and [^{14}C]-2-DG, resulting in inaccuracies in the operational equation that lead to an underestimation of LCMRglu (Schuier et al., 1990). Conversely, in severe hypoglycaemia (plasma glucose less than 4µmol/ml) the distribution volume of glucose is decreased and that of deoxyglucose is increased, leading to a marked increase in the value of the lumped constant and an overestimation of LCMRglu (Suda et al., 1990). Stress-induced or drug induced hyperglycaemia, therefore, are possible confounding errors in the calculation of LCMRglu. Therefore, within this work as several of the drug challenges used were known to induce hyperglycaemia animals were routinely fasted over-night in order to maintain normoglycaemia during the experiment. It is also important to note that fasting animals in this way did not result in severe hypoglycaemia (in saline-treated animals).

An additional criticism of the 2-DG technique has been that it does not account for the metabolism of 2-DG-P to 2-DG by glucose-6-phosphatase (Hawkins and Miller, 1987). However, the activity of glucose-6-phosphatase in cerebral tissue has been found to be low and rigorous neurochemical analysis has confirmed that the amounts present are unlikely to compromise the 2-DG method (Nelson et al., 1985; 1986; Sokoloff et al., 1977).

3.2 Experimental protocol for quantitative measurement of LCMRglu

Polyethene cannulae are surgically inserted into both the femoral arteries and veins of anaesthetised animals. Following surgery, animals are secured and lightly restrained by means of a plaster cast around the lower torso to a weighted block. Anaesthesis is then terminated and animals allowed to recover for 2 hours prior to further manipulation (i.e. injection of pharamacological agents) or [^{14}C]-2-DG injection. Measurement of LCMRglu is initiated by the intravenous (*i.v.)* injection of [^{14}C]-2-DG (7.4MBq.kg^{-1} in 2.5mls.kg^{-1} sterile saline) at a steady rate over 30 seconds. Over the subsequent 45 minutes of the experiment a series of 14 blood samples, approximately 0.75mls in volume, are collected into heparinised centrifuge tubes at pre-determined time intervals. In order to avoid hypovolaemia each blood sample is replaced by a similar volume of saline. The collection of samples is temporally distributed in such a way as to ensure full characterisation of the peak in plasma [^{14}C]-2-DG concentration during the first 5 minutes of the experiment and the full arterial profile of plasma [^{14}C]-2-DG over the course of the whole experimental period. Samples are immediately centrifuged and aliquots of plasma removed for the determination of plasma glucose and [^{14}C] concentrations by glucose oxidase assay and liquid scintillation analysis, respectively. At 45 minutes animals are rapidly killed by *i.v.* injection of sodium pentobarbitone and the brains rapidly dissected out, frozen (isopentane, -40^{o}C) and processed for quantitative autoradiography (Chapter 2, section 7.1). The three parameters determined by the experiment; the time-course of arterial [^{14}C]-2-DG concentration, time-course of arterial plasma glucose concentration and the tissue concentration of [^{14}C], as determined by autoradiography, are used in the operational equation of the technique to determine LCMRglu.

3.3 Semi-quantitative 2-deoxyglucose autoradiography

A semi-quantitative (SQ) 2-deoxyglucose method, modified from the fully quantitative method, has been applied to determine LCMRglu in transgenic mice (Kelly et al., 2002) and in mice in response to pharmacological challenge (Jordan et al., 2005). In this method measurement of LCMRglu is initiated in freely-moving conscious animals by the intraperitoneal (*i.p)* injection of [^{14}C]-2-DG (5μCi in 0.4mls) at a steady rate over 10 seconds. 42.5 minutes after the injection of [^{14}C]-2-DG animals are anaethetized in a perspex chamber containing halothane (2.5%) in a nitrous oxide:oxygen mixture (70%:30%) for 2.5 minutes. At exactly 45 minutes after isotope injection mice are rapidly decaptitated and a terminal blood sample collected by torso inversion. Terminal blood plasma [^{14}C] and glucose concentrations are determined from this sample by the methods previously outlined and the brains are processed as outlined for the quantitative 2-DG method. Autoradiographic image analysis allows for the determination of the [^{14}C] isotope concentration present in each brain ROI.

As the arterial plasma [^{14}C] and glucose concentration profile throughout the experimental period in each animal is not determined by this method the original analytical method developed by Sokoloff et al. (1977) can not be used to quantitatively define LCMRglu. Rather, LCMRglu is estimated as the ratio of the [^{14}C] isotope concentration in each ROI relative to that in a proposed control brain region, where LCMRglu is thought to remain constant between the experimental conditions. The constancy of LCMRglu in this region between experimental groups is implied by the observation of a similar [^{14}C] concentration in the standard region between experimental groups when the terminal plasma parameters ([^{14}C] and glucose concentration) also remain constant between groups. Observation of a similar relationship between the [^{14}C] concentration of the selected control region and the plasma [^{14}C]: [glucose] ratio of each animal, as displayed on an X-Y graph, within and between groups is also used to further implicate the validity of the standard region as an appropriate control. However, the relationship between these factors and any possible difference in this relationship between experimental groups is not usually analysed statistically by correlation or linear regression analysis, respectively. The control region is, however, often selected with reference to previous relevant data gained by the quantitative 2-DG method in the rat.

In these experiments the SQ was further modified from the reported method in two ways; (i) as the level of unphosphorylated [^{14}C]-2-DG has been reported to be significantly increased in the brain of rats at the experimental end-point following *i.p.* compared to *i.v.* injection (Kelly and Mcculloch, 1983a) animals in these studies were killed by rapid cervical dislocation and decapitation with no anaethesia in order to minimise the possible confounding influence of anaesthesia on the [^{14}C]-2-DG levels in the brain (ii) LCMRglu was estimated in line with a novel analytical method with increased validity (Chapter 3, study 1).

4. Ligand Binding Autoradiography

4.1 Receptor Theory

Ligand binding autoradiography experiments involve the interaction of a radiolabelled ligand with a target binding site to form a reversible ligand-receptor complex governed by the Law of Mass Action. A simple bimolecular reaction can be represented by the formula:

Equation 1

$$\text{Ligand} + \text{Receptor} \underset{K_{-1}}{\overset{K_{+1}}{\leftrightarrow}} \text{Ligand-Receptor Complex}$$

$$[L] + [R] \leftrightarrow [LR]$$

The Law of Mass Action states that the rate of a chemical reaction is proportional to the product of the effective concentrations of each participating molecule in that reaction. Therefore, if the concentration of participating molecules remains constant the reaction will eventually reach a state known as equilibrium, when the forward rate of the reaction is equal to the reverse rate of the reaction. In terms of radioligand binding this means that the rate of association (K_{+1}) between ligand and receptor becomes equal to the rate of ligand dissociation from (K_{-1}) the ligand-receptor complex.

The product of the above reaction [RL] is more commonly referred to as [B], the amount of the radioligand *bound* to the receptor. When equilibrium is attained the dissociation constant (K_d), which is a reflection of the affinity of the ligand for its binding site on the receptor, is given by:

Equation 2

$$K_d = [R].[L]/[B]$$

Numerically, K_d is equal to the concentration of the ligand that would be required to occupy 50% of the binding sites at equilibrium and is characteristic of both the ligand and the receptor. Therefore, if a ligand has a low K_d for a receptor binding site a low concentration of the ligand is required to occupy half of the receptor binding sites and the ligand is said to

display high affinity for the receptor. Ligands with low affinity have high K_d values and a large concentration is required to occupy 50% of the receptors.

At equilibrium, the total number of receptors present (Bmax) is equal to the number of receptors bound by the radioligand and unbound free receptors, represented as:

Equation 3

$$Bmax = [R] + [B]$$

Therefore, [R] may be substituted in equation 2 to give:

Equation 4

$$K_d = (Bmax - [B]).[L]/[B]$$

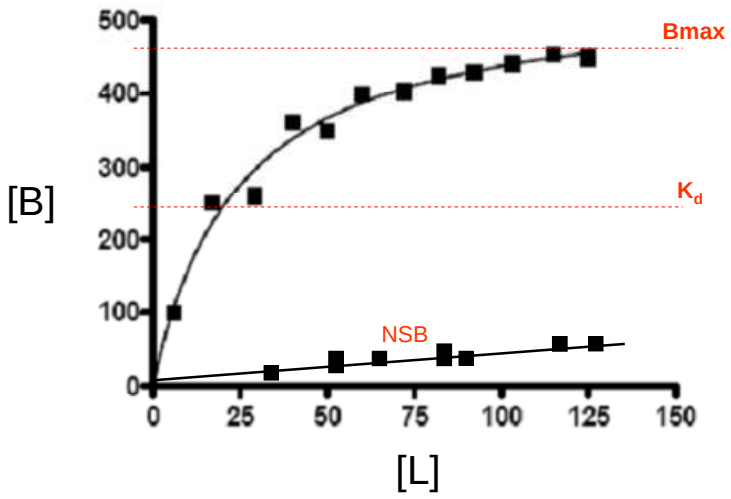
If specific binding follows the Hill-Langmuir equation then the relationship between the amount of bound ligand and the ligand concentration is:

Equation 5

$$[B] = Bmax\ [L]/\ [L]+ Kd$$

Saturation analysis methods can be used in order to determine estimates of both K_d and Bmax (Figure 2.6). [B] is a function of the radioligand concentration which increases in a rectangular hyperbolic fashion with [L]. As previously mentioned the dissociation constant (K_d) is equal to half of the maximum binding (1/2 Bmax) under conditions of equilibrium.

Figure 2.6 Saturation analysis



Non-specific binding (NSB), the binding of the ligand to components of the tissue other than the receptor is non-saturable and displays a linear relationship to [L]. Experimentally NSB may be determined by the displacement of the radioligand from a binding site by use of a non-labelled ligand specific to the site of interest.

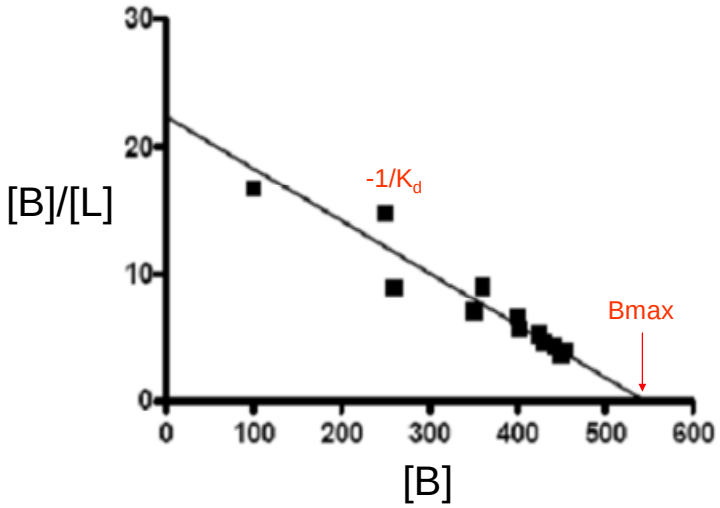
Equation 5 may be re-arranged to give:

Equation 6

$$[B] / [L] = (Bmax / K_d) + [B] / K_d$$

Bmax and K_d are more commonly estimated by Scatchard plot analysis of binding data, this directly relates to equation 6 and is shown in figure 2.7. In Scatchard analysis the concentration of [B]/[L] ligand is plotted directly against [B] giving a straight line with a gradient equal to $-1/K_d$ and the intercept of the line with the X-axis is equal to the Bmax (Scatchard, 1949). This analysis allows estimation of Bmax by extrapolation and thus avoids the need to use saturating concentrations of ligand. This method has been used extensively in the calculation of receptor binding kinetics in order to establish protocols for binding with each radioligand. The studies employed in this thesis employ kinetic analysis of ligand binding which has been calculated elsewhere (Table 2.3)

Figure 2.7 Scatchard plot



4.2 Receptor Autoradiography

Ligand binding autoradiography methods were used in order to obtain a complete pattern of SERT and serotonin receptor (5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A} and 5-HT_{2C}) binding throughout the brain in anatomically discrete regions. The receptor theory principles outlined above apply to all receptor autoradiography studies. The technique allows for the determination of the concentration of radioligand bound to a specific binding site (receptor/transporter) population in discrete brain areas. Brain sections are exposed to a buffer solution containing a known concentration of the radioligand until equilibrium is reached giving the total binding (TB) concentration of the radioligand in each brain region. Adjacent brain sections are also incubated in a solution containing exactly the same concentration of radioligand and under the same conditions except for the presence of a saturating concentration of a non-radiolabelled competitor (or non-specific determinant) for the binding site of interest. This allows for the determination of NSB, the concentration of radioligand bound to the section which is not directly bound to the binding site of interest. The concentration of radioligand bound to the binding site of interest is then calculated by subtracting the NSB concentration of radioligand in each brain region from the TB concentration of the radioligand in the same region.

4.2.1 Preparation of brain Sections for *in vitro* receptor autoradiography

For *in vitro* autoradiographic studies mice were killed by cervical dislocation followed by decapitation. The brain was then rapidly dissected out and frozen in isopentane at -40^{o}C and stored at -80^{o}C until sectioning. Prior to sectioning brains were frozen onto orientating microtome chucks with Lipshaw Embedding matrix and allowed to equilibrate to -20^{o}C in a cryostat for 30 minutes. 20μm coronal sections were cut on a Bright cryostat and thaw mounted onto Polysine coated slides (VWR, UK). Two sections, mounted onto separate slides, were taken for the duplicate determination of TB and the next adjacent section was taken on a separate slide for assessment of NSB. Three sections were taken every 60μm in the rostral to caudal direction. Sections were allowed to dry at room temperature and were then stored at -80^{o}C until the experimental day.

4.2.2 Method

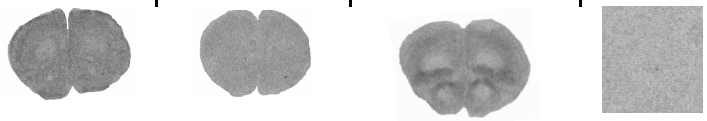
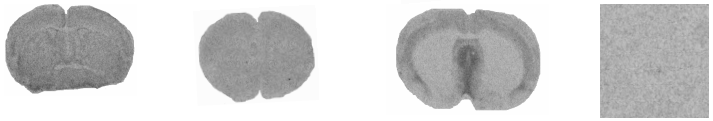
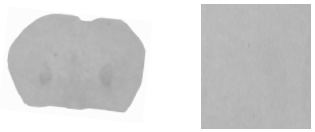
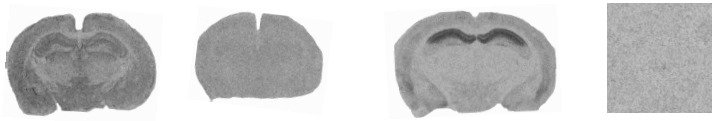
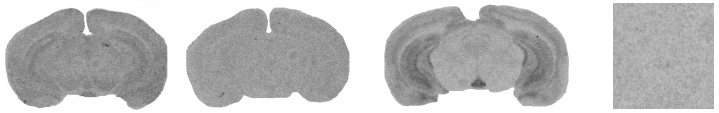
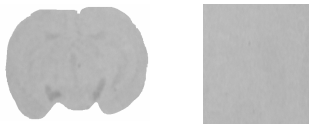
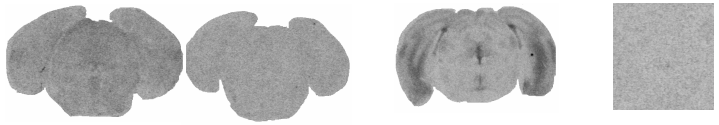
On the day of the experiment tissue sections were removed from the freezer and left to equilibrate to room temperature for 30 minutes. All autoradiographic incubations were completed in Coplin jars containing 70mls of assay buffer. The specific methodologies for each autoradiographic study is outlined in table 2.3. Briefly, sections were pre-incubated in assay buffer, then incubated in the presence of the [^{3}H]ligand for determination of TB or in the presence of the [^{3}H]ligand and a saturating concentration of 'cold' competitor ligand for determination of NSB, these incubations were then followed by a series of washes in fresh ice-cold (4^{o}C) assay buffer. Sections were then briefly dipped (5 seconds) in ice-cold distilled H_2O and excess liquid removed from the slide under a vacuum with a fine tipped Pasteur pipette then rapidly dried under a stream of warm air. Slides were then mounted onto autoradiographic cards and the sections opposed to [^{3}H]-sensitive film (Kodak, SB-5) along with pre-calibrated tritium standards ([^{3}H] microscales, Amersham) in light-tight cassettes for varying periods of time at -40^{o}C as specified in Table 2.3.

Table 2.3 Ligand binding autoradiography protocols

Target		SERT	5-HT$_{1A}$	5-HT$_{1B}$	5-HT$_{2A}$	5-HT$_{2C}$
Radioligand		[^{3}H]-Paroxetine	[^{3}H]-WAY100,635	[^{3}H]-GR 125,743	[^{3}H]-Ketanserin	[^{3}H]-Mesulergine
	~pKi	9.8	8.4	9	8.9	9
	Final Conc. (nM)	0.25	3	2	2	5
	Specific Activity (Ci/mM)	19.1	74	65	72.2	72
	Source	Amers.	Amers.	G.E.	P-E.	Amers.
NSB Determinant		Citalopram	5-HT	CP 93,129	DOI	DOI
	~pKi	8.7	9.4	8.5	9.2	8.6
	Final Conc. (µM)	4	10	1	1	1
	Source	S-A.	S-A.	S-A.	S-A.	S-A.
Protocol	Assay Buffer	50mM Tris (pH 7.7) + 120mM NaCl + 5mM KCl	50mM Tris (pH 7.4) + 10µM pargyline	50mM Tris (pH 7.4) + 4mM $MgCl_2$	170mM Tris (pH 7.7)	170mM Tris (pH 7.7)
	Pre-incubation	2 x 5 min. (25^{o}C)	2 x 5 min. (37^{o}C)	1 x 30 min (25^{o}C)	1 x 15 min. (25^{o}C)	1 x 15 min. (25^{o}C)
	Incubation	2 hours (25^{o}C)	1 hour (37^{o}C)	1.5 hours (25^{o}C)	2 hours (25^{o}C)	2 hours (25^{o}C)
	Washes	4 x 30 min. (4^{o}C)	1 x 2 min. + 1 x 3min. (4^{o}C)	3 x 10 min. + 1 x 5 min. (4^{o}C)	2 x 10 min. (4^{o}C)	2 x 10 min. (4^{o}C)
	Film exposure	6 weeks	6 weeks	6 weeks	6 weeks	10 weeks
	Additional Info.					100nM spiperone (S-A.) in incubations to occlude 5-HT$_{2A}$ binding
	Reference	De Souza et al., (1987)	Preece et al., (2004)	Wren (2000)	Preece et al., (2004)	Laakso et al., (1996)

Summary of Lingand binding autoradiography methods. For each target the [^{3}H]ligand, assay buffer constituents, non-specific binding (NSB) determinant and incubation protocols are outlined, along with appropriate references and additional relevant information for each assay. Abbreviations; Amers: Amersham, G.E.: G.E. Healthcare, P-E.: Perkin-Elmer, S-A.: Sigma-Aldrich. ~pKi values were gained from quoted references and the IUPHAR receptor database (www.iuphar-db.org, 2007).

Table 2.4 Representative autoradiograms from ligand binding studies

	[^{3}H]Paroxetine		[^{3}H]WAY 100,635		[^{3}H]Ketanserin		[^{3}H]Mesulergine		[^{3}H]GR 125,743	
	TB	**NSB**	**TB**	**NSB**	**TB**	**NSB**	**TB**	**NSB**	**TB**	**NSB**
[^{3}H] conc. range (nCi/mg)	0.10 – 5.66	0 – 1.52	0.20-12.05	0	1.63 – 13.17	1.23 – 6.74	1.81 – 14.01	1.19 – 5.33	0.83 - 0.36	0
Prefrontal Cortex										
Caudate										
Dorsal Hippocampus										
Ventral Hippocampus										
Raphé										

Representative autoradiograms showing the distribution of [^{3}H]Paroxetine, [^{3}H]WAY 100,635, [^{3}H]Ketanserin, [^{3}H]Mesulergine and [^{3}H]GR 125,743 binding from ligand binding studies. Autoradiograms taken from wild-type male mice. TB represents total binding while NSB represents non-specific binding. Where no image is presented TB was not distinguishable from NSB in any brain region at that level. The [^{3}H] concentration range (nCi/mg) for sections exposed to TB and NSB conditions for each radioligand is also shown. In [^{3}H}WAY 100,635 and [^{3}H]GR 125,743 binding NSB was below the level of quantification (0 nCi/mg).

5. Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assays (ELISA) allow for the detection and quantitative analysis of nanomolar (nM) and picomolar (pM) concentrations of proteins, hormones or drugs in biological fluids. The technique utilises the specificity of the antigen-antibody reaction with the compound of interest being the antigen and at least one of the antibodies targeted to the antigen being enzyme linked.

5.1. Detection of ACTH

A commercially available ACTH ELISA kit (Biomerica ACTH ELISA, IDS Ltd UK) was used in these studies to detect plasma concentrations of ACTH. This is a two-site ELISA designed to quantify the levels of intact (39 amino acid) biologically active ACTH with two polyclonal antibodies that recognize two different binding sites at opposite ends of the ACTH molecule. One antibody is a goat polyclonal antibody that binds to the carboxy-terminal of the (34-39 amino acids) ACTH molecule and is biotinyalated. During incubation the biotinylated antibody not only binds to ACTH molecules present in the solution but also binds the solid phase of the microplate well. The other antibody is a mouse antibody to ACTH which binds the mid-region and amino-terminus of ACTH (amino acids 1-24) and is conjugated with horseradish peroxidase (HRP). Both antibodies are present in excess of the concentration required to bind the ACTH present within the sample. During incubation both antibodies react with the ACTH molecules within the sample. However, only intact ACTH binds to both antibodies at the same time resulting in the formation of a “sandwich” complex. After incubation excess enzyme-linked antibody is removed from the well by repeated decanting and washing. Any ACTH bound to the solid-phase antibody remains in the well with the enzyme linked anti-body attached to the amino terminus. The “sandwich” complex is then incubated with the substrate tetramethylbenzidine (TMB) and the enzymic reaction then stopped by the addition of an acidic solution (sulphuric acid) resulting in the formation of a coloured product (yellow), the intensity of which is proportional to the concentration of ACTH within the sample.

5.1.1 Method

Plasma samples were stored at -80^{o}C until the day of analysis and were allowed to equilibrate to room temperature before use. 6 lyophilized ACTH calibrators of known concentration (range 0-515pg/ml ACTH) were reconstituted with distilled water as were two reference controls of known ACTH concentration, all of which were included in the kit. 200µl of each standard, the controls and each unknown plasma sample was added to a designated streptavidin coated well. 25µl of the biotinylated ACTH antibody (goat anti human ACTH) and 25µl of the enzyme-labelled (mouse monocolonal anti human ACTH) antibody solutions were then added to each well. The microplate was then covered with foil to avoid light exposure and incubated on an orbital shaker (170 rpm) for 4 hours at room temperature. The solution was then decanted from the microplate wells by inversion and each plate washed five times with working wash solution (in kit). 150µl TMB solution was then added to each well and the enzymatic reaction allow to incubate for 30 minutes at room temperature while on an orbital shaker (170rpm). The enzymatic reaction was stopped by the addition of 100µl stopping solution (1M sulphuric acid). The absorbance of each well was then measured using a microplate reader (Dynex technologies, MRX) at both 450nm and 405nm against 250µl of distilled water. The concentration of ACTH in each plasma sample was determined with reference to the calibration curve (concentration versus absorbance) generated by the in kit calibrators. The curve generated at 450nm was used to calculate ACTH concentrations up to 150 pg/ml while that at 405nm was used to analyse any ACTH concentrations above 150 pg/ml.

5.2 Detection of Corticosterone

A commercially available ELISA kit (IDS Ltd, UK) was used for the determination of plasma corticosterone concentrations in these studies. This kit is a competitive enzymeimmunoassay which utilised a polyclonal rabbit anti-corticosterone antibody coated onto the inner surface of polystyrene microtitre wells. Calibrators, controls and plasma samples are incubated for 24 hours with HRP labelled corticosterone. The wells are then washed and colour is developed by incubation with the chromogenic substrate TMB. The reaction is then stopped by the addition of a stop solution (0.5M hydrochloric acid) and the level of corticosterone in each sample is inversely proportional to the intensity of the colour in each well.

5.2.1 Method

Plasma samples were stored at -80°C until the say of the experiment and were allowed to equilibrate to room temperature before analysis. 6 lyophilized corticosterone calibrators of known concentration (range 0-133ng/ml corticosterone) were reconstituted with 1ml distilled water, as were two reference controls of known corticosterone concentration. Each plasma sample was diluted 1:10 with buffer (in kit, phosphate buffered saline with horse serum) prior to use. 100µl of each calibration standard, the controls and each unknown plasma sample was added to a designated antibody coated well. The lyophilised enzyme conjugate, containing the enzyme labelled corticosterone, was reconstituted with phosphate buffered saline (in kit) and then 100µl of the enzyme conjugate solution was then added to each well. The microplate was then sealed with an adhesive plate sealer and incubated for 24 hours at 4°C. After incubation the solution within the wells was decanted and the wells washed three times with wash solution (250µl per well, phosphate buffered saline with tween). After the final wash excess solution was removed from each well by inverting the microplate firmly on absorbent tissue paper. 200µl TMB solution was then added to each well and the incubation completed at room temperature for 30 minutes. The enzymatic reaction was then stopped by the addition of 100µl of stop solution (0.5M HCl) to each well. The absorbance of each well was then measured at 450nm, with reference to distilled water measured at 650nm, using a microplate reader within 30 minutes of the addition of the stop solution to the wells.

6. In situ hybridisation

In situ hybridisation (ISH) is a technique in which molecular biology and histochemical methods are combined to study gene expression in tissue sections. In brain sections this technique is particularly useful as it allows for the detection of gene expression, by detecting messenger RNA (mRNA) expression, while also preserving the cellular integrity of the tissue. This allows for the quantification of gene expression in distinct neuroanatomical localisations within the CNS.

ISH involves the annealing of a nucleic acid probe (in this thesis ^{35}S-radiolabelled riboprobes are used) with a sequence complementary to that of the mRNA of interest to the native mRNAs present within the tissue. The localisation of the probe can then be visualised, in the case of this thesis by autoradiographic methods. The riboprobes are generated by *in vitro* transcription from cloned DNA (cDNA) sequences for the target of interest which have been inserted into a transcription vector. In this vector, two RNA polymerase initiation sites exist that allow for the generation of both the anti-sense (complementary) and sense (same sequence) probes for the mRNA. As the sense probe has the same nucleotide sequence as that of the mRNA of interest it can be used in ISH as a control to detect any binding of the probe to the tissue which is not specific to mRNA hybridization.

6.1 Glucocorticoid (GR) and Mineralocorticoid (MR) ISH

For these studies mice were killed by cervical dislocation followed by decapitation. The brain was then rapidly dissected out and frozen in isopentane at -40^{o}C and stored at -80^{o}C until sectioning. Prior to sectioning brains were frozen onto orientating microtome chucks with Lipshaw embedding matrix and allowed to equilibrate to -20^{o}C in a cryostat for 30 minutes. Coronal sections at the level of the hypothalamic paraventricular nucleus (PVN) and dorsal hippocampus were thaw mounted onto poly-L-lysine coated slides and stored at -80^{o}C until the experimental day. In situ hybridisation studies were conducted as according to Seckl et al (1990) and Holmes et al (1995). Tissue sections were fixed in cold 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) containing 0.02% diethylpyrocarbonate for 10 minutes at room temperature. This was followed by 2 x 10 minute washes in 1 x PBS buffer, one wash in 0.1M triethanolamine (300ml) with 0.75ml of acetic anhydride, and a final 5 minute wash in 1 x phosphate buffered saline (PBS). Sections

were then dehydrated by sequential 2 minute dehydration in 70%, 80% and 90% ethanol washes.

Radiolabeled sense and anti-sense cRNA probes were transcribed from plasmids containing template fragments of complementary DNA (cDNA) for the rat GR (673 bp, PstI-EcoR1 fragment of rat cDNA) and MR (513 bp, EcoR1 fragment of rat cDNA) using ^{35}S-uridine-5'-trisphosphate (Amersham). 1µl of the required cDNA (1µg/µl) was added to an Rnase-free eppendorf with 9.9µl of reaction mix (Table 2.4). T7 RNA polymerase was used to generate the GR anti-sense cRNA probe and SP6 RNA polymerase was used in the generation of the anti-sense MR cRNA probe. SP6 and T7 RNA polymerase were used to generate the sense cRNA probes for GR and MR, respectively. Reactions using T7 were incubated at 37^{o}C for RNA probe generation, while those using SP6 were incubated at 40^{o}C. 1µl Dnase (Rnase free) was then added to the reaction mixture and further incubated for 15 minutes at 37^{o}C. The cRNA probes were then purified from the reaction mixture by use of a Nick column (G.E Healthcare) in 400µl TE buffer, and stored at -20^{o}C until use. The efficiency of probe transcription was confirmed by determining the amount of radioactivity in a 1µl sample of the probe by liquid scintillation analysis (~4000 cpm).

Table 2.5 GR and MR cRNA probe reaction mixture

	Volume (µl)
Transcription Buffer (5x concentrate)	2
1:1:1 ratio ATP, CTP, GTP mix	1
200mM DTT	0.5
Rnase Inhibitor	0.4
cDNA Template (µg/µl)	1
^{35}S-UTP	4
RNA Polymerase (T7 / SP6)	1
Total mixture volume	**9.9**

To reduce background binding of the radiolabbelled cRNA probe sections were exposed to a pre-hybridisation step. Sections were incubated in sealed boxes for 3 hours at 50^{o}C with 200µl per slide of pre-hybridisation buffer (50% deionised formamide, 1.2M NaCl, 0.02M Tris HCl, 2 x Denhardt's solution, 2µM EDTA, 1mg/ml denatured salmon sperm DNA and 0.2 µg/ml yeast transfer RNA).

Prior to hybridization incubation cRNA probes were denatured by heating to 75^{o}C for 10 minutes in hybridization mixture (10 x 10^{6} cpm/ml probe concentration; in 50% formamide, 1.2M NaCl, 0.02M Tris HCl, 2x Denhardt's solution, 0.2mg/µl denatured salmon sperm DNA, 0.2 µg/ml yeast transfer RNA, and 20% dextan sulphate hybridization mixture). 200µl per slide of the complete hybridization mixture was then added to the sections which were then incubated in sealed boxes over night (~16 hours). Following hybridization sections were washed three times in 2x SSC buffer for 5 minutes at room temperature before ribonuclease A digestion (0.09 µg/ml final concentration in 0.5M NaCl, 10µM Tris HCl, 1µM EDTA) for 1 hour at 37^{o}C. Sections were then serially washed in 2x SSC (60 mins, RT), 0.1x SSC (60 mins at 60^{o}C) and then a final 0.1x SSC wash (60 mins, 60^{o}C at start time and allowed to cool to RT) and then dehydrated in 50%, 70% and 90% ethanol in ammonium acetate (2 minutes for each concentration at RT). After dehydration slides were air dried overnight and exposed to autoradiographic film (Kodak, SB-5) for 5 days along with pre-calibrated [^{14}C] standards (Amersham).

7. Experimental Sample Analysis

7.1 ^{14}C, ^{3}H and ^{35}S Autoradiography

Radioactive isotopes, such as ^{3}H and ^{14}C and ^{35}S, have unstable nuclei and undergo radioactive decay in which they emit subatomic particles in an attempt to become more stable. In the case of the isotopes used in these studies electrons, also known as β rays, are emitted from the nucleus. The emission of these particles can be detected with x-ray autoradiographic film which has a photographic emulsion consisting of silver halide crystals surrounded by a capsule of gelatine. This allows each individual crystal of silver halide to act as an independent detector for emitted radioactive particles. Exposure of a silver halide molecule to radioactive particles causes a photochemical reaction in which silver ions are produced. This produces a stable-latent image on the autoradiographic film which can be visualised by chemical development. During the first stage of the development process the film is exposed to a reducing agent which converts silver ions into silver metal, while unexposed crystals remain as silver halide. During the second stage of the developing process the film is exposed to a fixing agent that dissolves silver halide, removing it from the film, but not the silver metal. This results in the production of spatially accurate "negative image" of the brain section, in which the area of the film exposed to the greatest number of radioactive particles has the highest density of opaque silver atoms. The isotope concentration in the source of the radiation can then be determined by measuring the optical density (O.D) of opaque silver atoms within a defined area.

7.1.1 Densitometric Analysis

Autoradiographic images on x-ray film were analysed using a computer bacsed image analysing system (MCID, Imaging Research Inc.). The O.D of pre-caibrated standards exposed to the film in concurrence with the brain sections was first measured, which allowed for the construction of the calibration curve for isotope concentration against O.D. [^{3}H]-standards with tissue equivalent [^{3}H] concentrations of 0.1-110 nCi/mg (Amersham International, UK) were used in the analysis of the ligand binding autoradiography studies, as tritiated ligands were always used. In both the [^{14}C]-2-deoxyglucose and in-situ hybridization studies, using [^{35}S], pre-calibrated [^{14}C] standards with tissue equivalent [^{14}C] concentrations of 40-1098 nCi/mg (Amersham International, UK) were used. Use of [^{14}C] standards to quantify [^{35}S] concentrations by autoradiographic methods is deemed

appropriate due to the linear relationship between these isotopes of concentration with O.D (Miller, 1991). The O.D of discrete brain regions, as identified with reference to a stereotaxic atlas of the mouse brain (Franklin and Paxinos, 1997), were then determined.

For each animal the mean O.D of each discrete brain region was deteremined from a total of 6 to 12 bilateral measurements, dependent upon the size of the regions being measured, in 3 to 6 sequential brain sections. For each brain region O.D measurements were detemined in a circular field of pre-determined size that fitted easily in to the ROI. The size of the measurement field for each ROI was maintained between all experimental groups and conferred the advantage of avoiding the possible influence of any artefacts or tissue damage on O.D measurement. The isotope concentration of each discrete brain region was then determined from the mean O.D of that region with reference to the calibration curve generated by the co-exposed standard (isotope concentration versus O.D). Analysis of all autoradiograms was completed blind to each animals genotype and/or treatment group.

In [^{14}C]-2-deoxyglucose experiments this data was used within the [^{14}C]-uptake ratio operational equation, together with the blood data collected for each animal in order to determine the [14C]-uptake ratio in each ROI (Chapter 3, Study 1). In ligand binding autoradiography experiments determination of isotope concentration for each brain ROI was carried out on consecutive brain sections for the determination of TB, in duplicate, and NSB. Specific binding could then be calculated by subtracting the value for NSB from that of TB. In in-situ hybridisation studies isotope concentrations determined from sense exposed sections were subtracted from those of anti-sense exposed sections.

7.2 Liquid Scintillation Analysis

In [^{14}C]-2-deoxyglucose experiments blood plasma [^{14}C] isotope concentration was detected by liquid scintillation analysis (LSA), an analytical technique that calculates the kinetic energy of nuclear emission from a radiolabelled sample by measuring the rate of light photon emission from a liquid sample. A vial containing the radiolabelled sample (20µl plasma) and scintillation cocktail (5mls) is placed into a scintillation counter which allows photon intensity to be observed. The beta particles emitted from the radioactive isotope first transfer their energy to aromatic solvent molecules and then fluor molecules in the cocktail, which dissipate this energy by emitting visible light (photons). These photons are detected by photonmultiplier tube (PMT) of the scintillation counter, which converts the frequency of

photon detection into an electrical impulse. The amplitude of the electrical impulse is directly proportional to the number of photons detected within the sample. The amount of radioactivity in the sample is then automatically calculated by counting the number of disintegrating atoms in the sample over a given period of time.

8. Specific Experimental Methodology

8.1 Study 1- Validation of semi-quantitative 2-deoxyglucose autoradiography

The validity of the current (Jordan et al., 2005; Kelly et al., 2002) and a novel analytical method for determination of LCMRglu by semi-quantitative [^{14}C]-2-DG was assessed by comparison of the results gained by these analytical methods to that of the quantitative method. These analyses were completed on data originally obtained by the quantitative method investigating the LCMRglu response to 15mg.kg^{-1} *i.p.* 3,4-methylenedioxymethamphetamine (MDMA) in male Dark-Agouti rats in 71 diverse ROI (Quate et al, 2004).

8.2 Study 2- Constitutive brain function in *h*SERT over-expressing mice

The density of SERT in 46 brain ROI was determined in male and female *h*SERT OVR mice and their Wt littermates (n = 6, each genotype and sex) by quantitative [^{3}H]-paroxetine binding autoradiography. Constitutive brain function was assessed by determining LCMRglu in 47 ROI in both male and female hSERT OVR mice and their Wt littermates by use of the SQ [^{14}C]-2-deoxyglucose autoradiographic technique. Determination of LCMRglu in females was spread over the reproductive cycle (4 days) to control for the known impact of the cycle on LCMRglu (Nehlig et al., 1985). Each animal received 0.2mls of saline *i.p.*10 minutes prior to the injection of [^{14}C]-2-DG *i.p.* (*h*SERT OVR; male Wt: n= 11, OVR: n= 11, female Wt n= 12, OVR n= 12). These groups were derived from those animals used as controls in additional LCMRglu experiments investigating the effects of the various pharmacological challenges on LCMRglu in Wt and *h*SERT OVR mice.

Statistics

Data from both the [^{3}H]-paroxetine binding and constitutive LCMRglu studies were analysed using 2-way ANOVA with gender (male, female) and genotype (Wt, *h*SERT) as the dependent variables. Within the LCMRglu study a significant genotype effect was analysed within gender by 2-way ANOVA with Bonferroni *post-hoc* correction for multiple comparison. Significance was set at $p<0.05$ throughout.

8.3 Study 3- 5-HT_{1A} function in *h*SERT over-expressing mice

5-HT_{1A} binding was assessed in 48 ROI in male and female *h*SERT OVR mice (n = 6 each gender) and their Wt littermates (n = 6 each gender) by quantitative [^{3}H]WAY 100,635 binding autoradiography. LCMRglu was determined in 47 brain ROI by semi-quantitative 2-DG autoradiography under basal conditions with [^{14}C]-2-DG injected *i.p*. 10 minutes after saline injection (0.2ml, *i.p*) in Wt and hSERT OVR mice of both sexes (male: Wt n= 11, OVR n= 11; female Wt n= 12, OVR n= 12). 5-HT_{1A} function was assessed in Wt and hSERT OVR mice of both sexes by observing the LCMRglu response in the same 47 brain regions to 10mg.kg^{-1} 8-OH-DPAT (*i.p*, 0.2mls), with 2-DG being injected 10 minutes after the acute 8-OH-DPAT injection (male: Wt n= 9, OVR n= 9, female Wt n= 9, OVR n= 10). The ability of this treatment protocol to produce pronounced alterations in LCMRglu, as assessed by the SQ [^{14}C]-2-DG, was determined in a set of initial dose-response experiments (1, 5 and 10mg.kg^{-1}, n = 4 each treatment group) completed in male and female wild-type male mice. In these initial experiments it was decided that [^{14}C]-2-DG would be injected 10 minutes after the 8-OH-DPAT (*i.p*) injection as the behavioural effects of the drug (head-weaving, hyperlocomotion and forepaw treading) were fully manifest at this time. In females all [^{14}C]-2-DG experiments and the harvesting of brains for [^{3}H]WAY100,635 binding studies were spread across the reproductive cycle in order to control for the confounding influence of the cycle on LCMRglu and 5-HT_{1A} pharmacology and function (Maswood et al., 1995; Osterlund et al., 2000; Uphouse et al., 1991). In [^{14}C]-2-DG experiments animals were randomly allocated to their treatment group on the day of the experiment.

Statistics

Data (mean ± s.e.m) from the [^{3}H]WAY 100,635 study were analysed using 2-way ANOVA with gender (male, female) and genotype (Wt, *h*SERT OVR) as the dependent variables. Bonferroni post-hoc correction for multiple comparison was applied when analysing for the effect of *h*SERT OVR on binding within gender. LCMRglu data were also analysed using ANOVA. The significance of the 8-OH-DPAT response was analysed by 2-way ANOVA (within genotype) with Bonferroni post-hoc correction for multiple comparisons. Gender x 8-OH-DPAT interactions were also analysed by 2-way ANOVA (within genotype). The significance of gender x genotype, genotype x 8-OH-DPAT and gender x genotype x 8-OH-DPAT interactions were analysed using Univariate ANOVA. Significance was set at $p<0.05$ throughout.

8.4 Study 4- 5-$HT_{2A/C}$ receptor function *h*SERT over-expressing mice

5-HT_{2A} binding was assessed in 50 brain ROI in both male and female *h*SERT OVR mice (n = 6, each sex) and their Wt littermates (n = 6, each sex) by quantitative [^{3}H]Ketanserin binding autoradiography. In the same animals 5-HT_{2C} pharmacology was characterised in 43 ROI by quantitative [^{3}H]mesulergine autoradiography. LCMRglu was determined in 47 brain regions by semi-quantitative [^{14}C]-2-DG autoradiography under basal conditions with [^{14}C]-2-DG injected 15 minutes after saline injection (0.2ml, i.p) in Wt and hSERT OVR mice of both sexes (male: Wt n= 11, OVR n= 11; female Wt n= 12, OVR n= 12). 5-$HT_{2A/C}$ function was assessed in Wt and hSERT OVR mice of both sexes by observing the LCMRglu response to 25mg.kg^{-1} DOI (*i.p*, 0.2mls) in the same 47 brain regions, with [^{14}C]-2-DG being injected 15 minutes after the acute DOI injection (n= 8 for each genotype and sex). This treatment protocol had previously been confirmed to induce alterations in LCMRglu in rats (Freo et al., 1991; 1992) in quantitative [^{14}C]-2-DG autoradiography and was confirmed to produce pronounced alterations in LCMRglu, as assessed by the SQ [^{14}C]-2-DG method, in initial dose-response experiments completed in male and female wild-type mice (dose 2.5, 10 and 25mg.kg^{-1}; n = 4 animals per group). In females all [^{14}C]-2-DG experiments and the harvesting of brains for the ligand binding studies were spread across the reproductive cycle in order to control for the confounding influence of the cycle on LCMRglu and 5-$HT_{2A/C}$ function and pharmacology (DiazVeliz et al., 1997; Sumner and Fink, 1998). In [^{14}C]-2-DG experiments animals were randomly allocated to their treatment group on the day of the experiment.

Statistics

Data for [^{3}H]ketanserin and [^{3}H]mesulergine binding were analysed using 2-way ANOVA with gender (male, female) and genotype (Wt, *h*SERT) as the dependent variables. A significant genotype effect on binding was analysed within gender by 2-way ANOVA with application of *post*-hoc Bonferroni correction for multiple comparison. Data from LCMRglu studies were also analysed using ANOVA. The DOI effect was analysed by 2-way ANOVA (within genotype) with Bonferroni post-hoc correction for multiple comparison. The genotype effect, gender x DOI interaction, genotype x DOI interaction and the gender x genotype x DOI interaction were analysed using Univariate ANOVA. Significance was set at $p<0.05$ throughout.

8.5 Study 5- 5-HT_{1B} function in *h*SERT over-expressing mice

5-HT_{1B} binding was assessed in 3 ROI in male and female *h*SERT OVR mice (n = 6 in each gender) and their wild-type litter mates (n = 6 in each gender) by quantitative [^{3}H]GR 125,743 binding autoradiography. LCMRglu was determined by SQ [^{14}C]-2-DG autoradiography under basal conditions, with 2-DG injected *i.p.* 10 minutes after saline injection (0.2ml, *i.p.*), in Wt and hSERT OVR mice of both sexes (male: Wt n= 11, OVR n= 11; female Wt n= 12, OVR n= 12). 5-HT_{1B} function was assessed in Wt and *h*SERT OVR mice of both sexes by observing the LCMRglu response to 10mg.kg^{-1} CP 94,253 (0.2mls, *i.p.*) with [^{14}C]-2-DG being injected 10 minutes after the acute CP 94, 253 injection (male: Wt n= 9, OVR n= 10, female Wt n= 9, OVR n= 9). The ability of this treatment protocol to produce alterations in LCMRglu, as assessed by the SQ [^{14}C]-2-DG, was determined in a set of initial dose-response experiments (1, 5 and 10mg.kg^{-1}, n = 4 each treatment group) completed in male and female wild-type mice. In females all [^{14}C]-2-DG experiments and the harvesting of brains for [^{3}H]GR 125,743 binding studies were spread across the reproductive cycle in order to control for the confounding influence of the cycle on LCMRglu and the possible influence in 5-HT_{1B} pharmacology and function. In [^{14}C]-2-DG experiments animals were randomly allocated to their treatment group on the day of the experiment.

Statistics

Data from the [^{3}H]GR 125,743 binding study were analysed using 2-way ANOVA. LCMRglu data were also analysed using ANOVA. The significance of the CP 94,253 effect was analysed using 2-way ANOVA (within genotype) with Bonferroni post-hoc correction for multiple comparison. Genotype effects and the genotype x CP 94,253 interaction were also analysed using 2-way ANOVA (within gender). Gender x CP 94,253, genotype x CP 94,253 and the gender x genotype x CP 94,253 interaction were analysed using Univariate ANOVA. Significance was set at $p<0.05$ throughout.

8.6 Study 6- Stress axis function in male *h*SERT over-expressing mice

Basal plasma ACTH and corticosterone levels were determined in male hSERT OVR (n=8) and their Wt (n=8) littermates by radioimmunoassay. Stress axis responsiveness was determined in male hSERT OVR (n=8) and Wt mice (n=8) by observing plasma ACTH and corticosterone levels 15 minutes after acute saline injection (0.2mls, *i.p.*). The influence of specific 5-HT receptor subtypes on the activity of the HPA axis was assessed in *h*SERT OVR and Wt mice by determining plasma ACTH and corticosterone levels 15 minutes after injection with 0.1mg.kg^{-1} 8-OH-DPAT (0.2mls, *i.p.*, Wt: n=8, *h*SERT OVR n=8), 10mg.kg^{-1} CP 94,253 (0.2mls, *i.p.*, Wt n= 8, *h*SERT OVR n = 8) and 2.5mg.kg^{-1} DOI (0.2mls, *i.p.*, Wt n= 8, *h*SERT OVR n = 8) to characterised the influence of 5-HT$_{1A}$, 5-HT$_{1B}$ and 5-HT$_{2A/C}$ receptors respectively. The doses of 8-OH-DPAT and DOI used in these studies have previously been found to result in peak plasma levels of ACTH and/or corticosterone secretion at 15 minutes post-injection (8-OH-DPAT: Li et al., 1999; DOI: Van de Kar et al., 2001; Bagdy et al., 1996). As no data are available upon the influence of the 5-HT1B agonist CP 94,253 on ACTH and corticosterone secretion the dose administered was chosen to coincide with that used in our LCMRglu studies (10mg.kg^{-1}). The 15 minute time-point for culling was chosen to coincide with available controls (saline-treated animals) and with the knowledge that central 5-HT$_{1B}$ receptor activation results in peak plasma ACTH concentrations at this time (Van de Kar et al., 1994). All studies of stress axis function were completed between 8.00am-10.30am to limit the influence of circadian variation in plasma ACTH and corticosterone levels. All animals were randomly assigned to the respective treatment group on the day of the experiment. Hormone levels were detected by enzyme-linked immunoassay as previously outlined.

Central glucocorticoid (GR) and mineralocorticoid (MR) receptor mRNA levels were determined in key brain regions implicated in the control of HPA axis function including the PVN and dorsal hippocampus, of male *h*SERT OVR (n= 6) and wild-type (n= 6) littermates by in-situ hybridisation.

Statistics

ACTH and corticosterone data were analysed using 2-way ANOVA with genotype (Wt, *h*SERT OVR) and treatment (basal, saline, 8-OH-DPAT, DOI, CP 94,253) as the dependent variables. Statistical analysis of the effect of acute mild stress (saline injection) was completed relative to hormone levels under basal conditions, while the effect of acute drug

treatment (8-OH-DPAT, DOI, CP 94,253) were analysed relative to acute saline treated animals. GR and MR binding data were analysed using Student's t-test. Acceptable levels of significance were set at $p<0.05$ throughout.

Chapter 3 - Results

1. Study 1- Validation of semi-quantitative 2-deoxyglucose autoradiography

1.1 Rationale

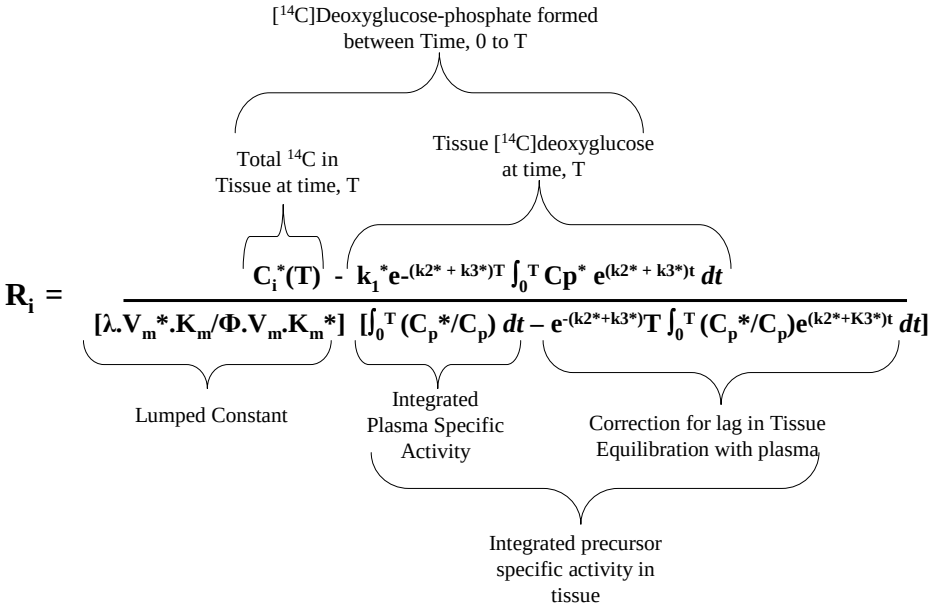
The uptake of radiolabelled 2-deoxyglucose into brain tissue is influenced by a number of factors; the metabolic activity of brain cells, which constitutes the factor of interest as it provides an index on neuronal activity and brain function, the amounts of tracer available in the blood compartment over the time of the experiment, and the levels of endogenous blood-borne glucose with which the tracer competes for transport into the brain compartment. Thus, the original fully quantitative approach developed by Sokoloff et al. (1977) requires that the animal be prepared surgically to allow for the intermittent sampling of arterial blood from which tracer and glucose concentrations can be measured and applied to the operational equation. While this fully quantitative approach is undoubtedly very powerful, the intravascular cannulation required does impose restrictions upon the experimental design and limits the usefulness of the technique. A number of attempts have been made to circumvent these limits, either by ignoring the inter-animal variation in plasma tracer and glucose levels, or by generating blood data that give an approximation to plasma histories of tracer and glucose concentrations.

A semi-quantitative (SQ) [^{14}C]-2-DG autoradiographic imaging technique has been proposed previously for the measurement of LCMRglu as an index of neuronal function in mice (Jordan et al., 2005; Kelly et al., 2002). In contrast to the quantitative technique the SQ method involves the intraperitoneal (*i.p*) rather than intravenous (*i.v)* injection of [^{14}C]-2-DG and the collection of a single terminal blood sample as opposed to the collection of 14 timed arterial samples throughout the experimental time period. These modifications were driven by the fact that the required cannulation of femoral arteries and veins is technically difficult in mice and that the removal of the blood volume required in the quantitative technique may induce hypovolaemia in mice, invalidating LCMRglu determination. Furthermore, use of the SQ method confers the advantage of allowing behavioural observation during the determination of LCMRglu as animals are not restrained during the experimental period.

In the SQ method the concentration of [^{14}C] and glucose in the terminal blood sample are expressed as a ratio ([^{14}C] / [glucose]) for each animal and this is assumed to be an accurate

reflection of the plasma [^{14}C] and [glucose] profiles throughout the experimental period. Animals in which this ratio can be identified as an "outlier" from the overall group mean can be excluded from further analysis. Subsequently, LCMRglu can be estimated in each brain region of interest (ROI) by deriving the ratio of [^{14}C] in that ROI relative to that of a selected reference region, where the rate of LCMRglu is assumed to be constant both within and between the experimental groups. A consistent rate of LCMRglu in the reference region is usually implied by the observation of a relatively constant [^{14}C] concentration in this region between experimental groups under conditions in which the plasma parameters also remain constant. However, many experimental manipulations – physiological, pharmacological or pathological – have an effect upon circulating glucose levels that could generate marked intergroup differences in the uptake of tracer into the reference brain region. Although an element of objectivity can be introduced by examining the relationship between the [^{14}C] concentration in the reference region and the terminal plasma [^{14}C] / [glucose] ratio between experimental groups, claims that this relationship remains constant between groups have never been tested statistically in previous publications. It has been assumed that minor individual variations from the overall group and intergroup relationships have only minor effects upon the certainty of the data, but again this has never been tested.

In these initial studies we have investigated the validity of the SQ 2-deoxyglucose methods as currently published with particular attention to the assumption of a consistent rate of LCMRglu in the selected reference region and the influence that the choice of an inappropriate reference region could have upon the estimation of LCMRglu when using SQ analysis. From these studies we identified limitations in the current SQ analytical method and with these in mind developed and tested a novel form of analysis, [^{14}C]-uptake ratio ([^{14}C]-UR) analysis, based upon direct modification of the operational equation used in the quantitative method. Use of this novel form of analysis allows for the direct estimation of LCMRglu within each brain region by comparing the amount of radioactivity in that region (C_i*) to the terminal plasma variables (plasma [^{14}C]-2-DG (Cp*) and [glucose] (Cp) concentrations). This ratio is then adjusted in accordance with the plasma variables of each animal to limit the influence of variability in the plasma parameters between animals on LCMRglu estimation.



Operational Equation for quantitative 2-deoxyglucose autoradiography. For abbreviations see those in legend of figures 2.4 and 2.5.

The complexity of the operational equation is much reduced by the fact that as the time following acute administration of [^{14}C]-2-DG increases, and the concentration of [^{14}C]-2-DG in the plasma approaches zero, those factors containing the rate constants (k1*, k2* and k3), the correction for lag in the equilibration between tissue and plasma and the concentration of unphosphorylated [^{14}C]-2-deoxyglucose in the tissue, also approach zero. Indeed, at infinite time these values will become zero but even at 45 minutes following a pulse of [^{14}C]-2-DG their contribution is deemed insignificant (Sokoloff et al., 1977). Therefore, we propose that for LCMRglu estimation in the SQ method these factors may be removed from the equation as long as an appropriately long time period following tracer injection is used. This gives:-

$$R_i = \frac{C_i^*(T)}{[\lambda.V_m^*.K_m/\Phi.V_m.K_m^*]\ [\int_0^T (C_p^*/C_p)\, dt]}$$

Equation 1

In the SQ method we do not have the detailed profile of the plasma [^{14}C]-2-DG and [glucose] history throughout the experimental time period and therefore we assume that the

concentration of these factors in the terminal sample provides an accurate reflection of the plasma history. Therefore, this equation can further be simplified to:-

$$R_i = \frac{C_i^*(T)}{[\lambda.V_m^*.K_m/\Phi.V_m.K_m^*]\ (C_p^*/C_p)}$$

Equation 2

The lumped constant is proposed to remain the same between all brain regions and animals and so this factor may also be removed from the equation in order to further simplify analysis. In addition, under normal physiological conditions the values of the lumped and rate constants for [^{14}C]-2-DG uptake and metabolism are consistent between animals. Therefore, any variability in the plasma [^{14}C]-2-DG and glucose histories between animals is the primary factor dictating variability in the level of [^{14}C] measured in tissues where the metabolic rate is constant. This means that adjustment may be made to the ratio gained from equation 2 in accordance with the plasma [^{14}C]-2-DG / [glucose] ratio of each individual animal relative to both the other animals within the same experimental group and also between experimental groups. This gives the final equation for [^{14}C]-uptake ratio analysis:-

$$R_i = \left[\frac{C_i^*(T)}{(C_p^*/C_p)}\right]\left[\frac{(Cp^*/Cp)i}{\mu(C_p^*/C_p)g}\right]\left[\frac{\mu(C_p^*/C_p)g}{\mu(C_p^*/C_p)cg}\right]$$

Operational equation for [^{14}C]-uptake ratio ([^{14}C]-UR) analysis in semi-quantitative 2-deoxyglucose autoradiography. Ci(T) represent total tissue [^{14}C] concentration. Cp* represents terminal plasma [^{14}C]-2-DG concentration. Cp represents terminal plasma glucose concentration. (Cp*/Cp)i represents the plasma ratio within an individual animal, whereas μ(Cp*/Cp)g represents the mean plasma ratio of that animals experimental group and μ(Cp*/Cp)cg the mean plasma ratio of the chosen control group. For analysis in which the animal belongs to the control group the final factor in this equation equals 1.*

1.2 Methods

In order to assess the validity of both our new approach to quantifying LCMRglu and the SQ methodology, as applied previously, we examined the effects of 3,4-methlyendioxymethamphetamine (MDMA) upon LCMRglu in male Dark Agouti (DA) rats as measured by these two approaches relative to data generated using the fully quantitative method. The fully quantitative data were obtained from reanalysis of [^{14}C]-2-DG autoradiography experiments published previously from this laboratory by Quate et al. (2004). Briefly, LCMRglu was determined in 71 ROI in male DA rats treated with saline (n = 5) or 15mg.kg^{-1} *i.p* MDMA (n = 5). Measurement of LCMRglu was initiated 15 minutes after the injection of saline or MDMA. A computer based image analysis system (MCID/M5+) was used to determine the local tissue [^{14}C] concentration in each ROI from the optical density of autoradiographic brain images relative to [^{14}C]-standards (Amersham, UK).

From the raw experimental data LCMRglu was first determined quantitatively using the operational equation and the complete recorded plasma history profile with the tissue [^{14}C] concentration of each brain region generated from reanalysis of the existing autoradiograms (Sokoloff et al., 1977). Two approaches to the original SQ analysis were applied to the data, the first with a reference region shown by quantitative analysis to have a constant LCMRglu between experimental groups (dorsal tegmental nucleus, DTN) and the second with a region shown to have significantly increased LCMRglu in MDMA-treated animals (corpus callosum, CC). This allowed us to both assess the validity of the methods employed in SQ analysis to verify the reference region as an appropriate standard, and to determine the possible impact of an actual alteration in LCMRglu in the reference region upon SQ LCMRglu determination. The validity of each reference region as an appropriate control was investigated in line with previously reported methods (Kelly et al., 2002), but in addition we also applied linear regression and correlation analysis to statistically investigate the relationship between the [^{14}C] concentration in the reference region and the plasma ratio within and between experimental groups. LCMRglu was then estimated as the ratio of [^{14}C] in each ROI to that of the chosen reference region in each animal. The [^{14}C]-uptake ratio analysis method outlined above was also applied to the same data set to assess the validity of this novel method to estimate LCMRglu.

To investigate the validity of the assumption that terminal plasma [^{14}C] / [glucose] ratios provide as accurate estimation of the plasma profile history of any given animal we

performed a correlation analysis of the relationship between the terminal plasma ratio in each animal and the area under the curve (integral) derived from the 14 times plasma samples. Within each animal the [^{14}C] / [gluocose] ratio of each plasma sample was first obtained and the integral then determined from the X-Y plot of this ratio against time. Correlation analysis was completed on the relationship between the terminal plasma ratio and the integral given for the plasma history profile of each animal.

In each form of analysis LCMRglu data (mean ± s.e.m) were analysed using student's t-test with significance set at $p<0.05$. The validity of each analytical method was assessed by comparing the magnitude, directionality, localisation and statistical outcome of alterations in LCMRglu detected between saline and MDMA-treated animals compared to those detected by the quantitative method.

1.3 Quantitative determination of LCMRglu

Quantitative analysis revealed the MDMA produced significant changes in LCMRglu in 21 of the 71 ROI analysed, in comparison to saline controls. Of these 21 alterations 19 represented significant increases in LCMRglu (range 19% to 88%) and 1 represented a significant decrease in LCMRglu, detected in the posterior cingulate cortex (-19%). These alterations were consistent with previously reported MDMA-induced alterations in LCMRglu (Wilkerson and London, 1987). In quantitative analysis LCMRglu was found to be significantly increased in the corpus callosum (+19%) of MDMA-treated animals relative to saline controls, whereas LCMRglu in the DTN (-5%) was not significantly altered. Detailed data from quantitative analysis are shown in tables 3.1.2 to 3.1.7.

1.4 Semi-quantitative determination of LCMRglu

1.4.1 Validation of the control region

In contrast to the findings from quantitative analysis, the rate of LCMRglu was found to be constant in both the DTN and corpus callosum between experimental groups as defined by the terms used in SQ analysis as there was no significant difference between any of the terminal plasma parameters and no significant difference in the mean [^{14}C] concentration of these regions between experimental groups. In addition, linear regression analysis also confirmed that the relationship between [^{14}C] concentration in these regions and the plasma

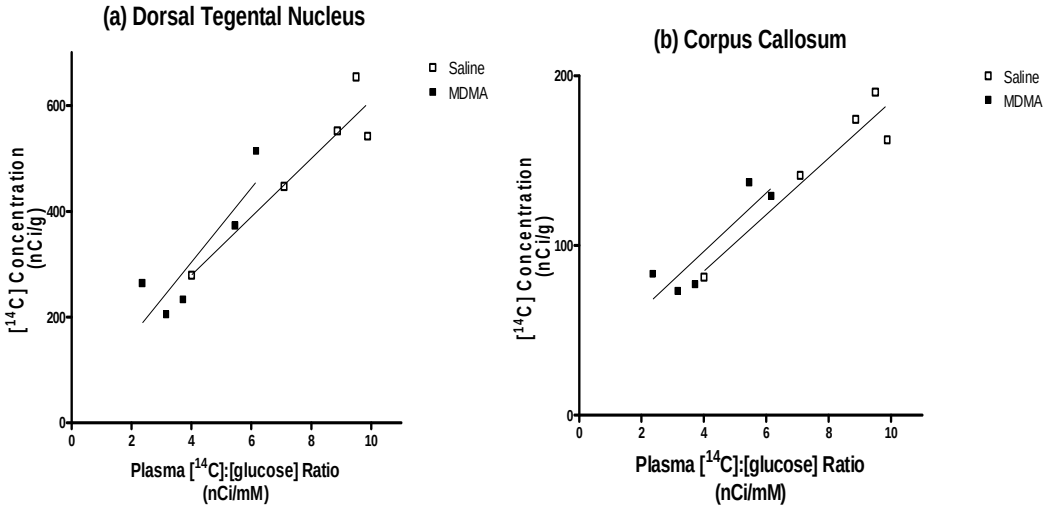
ratio was not significantly different between experimental groups in terms of slope, intercept or elevation (Table 3.1.1). Furthermore, the [^{14}C] concentration of these regions showed a significant correlation with the plasma ratio in both experimental groups (Figure 3.1.1).

Table 3.1.1 Control regions and plasma parameters in SQ analysis

	Saline	MDMA
Plasma [^{14}C] concentration (nCi/ml)	93.7 ± 20.8	55.2 ± 5.5
Plasma glucose concentration(mg/ml)	12.2 ± 1.5	11.9 ± 1.4
Plasma [^{14}C]:[glucose] ratio (nCi/mg)	7.4 ± 1.1	4.8 ± 0.5
Dorsal Tegmental Nucleus [^{14}C] (nCi/mg)	493.8 ± 63.1	316.8 ± 56.7
Corpus Callosum [^{14}C] concentration (nCi/mg)	146.5 ± 21.6	126.2 ± 23.5
Linear regression analysis		
Dorsal Tegmental Nucleus		
Intercept	58.31 ± 92.32	22.66 ± 96.20
Slope	55.24 ± 11.30	70.30 ± 21.77
Corpus Callosum		
Intercept	18.22 ± 26.2	27.9 ± 22.4
Slope	16.6 ± 3.2	17.2 ± 5.1
Correlation analysis		
Dorsal Tegmental Nucleus R^2	0.89*	0.78*
Corpus Callosum R^2	0.89*	0.79*

*Plasma data and control region validity in saline and MDMA-treated DA rats. Data presented as mean ± s.e.m. *indicates significant correlation(P<0.05).*

Figure 3.1.1 Control region-plasma ratio relationships



Relationship between tissue [^{14}C] (nCi/mg) concentration in the selected reference region (a) dorsal tegmental nucleus (b) corpus callosum, and terminal plasma [^{14}C] / [glucose] concentration ratio in saline and MDMA-treated DA rats. In each control regions there was no significant difference in the relationship between [^{14}C] concentration and the plasma [^{14}C]:glucose ratio between the experimental groups (linear regression).

1.4.2 LCMRglu

When the DTN was used as the reference region significant alterations in LCMRglu were detected in 17 of the 71 ROI. All of these significant alterations were also found from quantitative analysis and the directionality and magnitude of significant LCMRglu alterations was also maintained. However, there were 3 “false- negative” results where this approach failed to identify significant alterations in LCMRglu which were detected by the quantitative method. When the corpus callosum was used as the selected reference region the magnitude, localisation and directionality of detected LCMRglu alterations was changed in comparison to the quantitative method (see Figure 3.1.4 for representative examples). In this case significant alterations were found in 27 of the 71 ROI analysed, that is 10 of these significant alterations were “false-positives” giving a statistically significant alteration in LCMRglu in a ROI which was not found in quantitative analysis. All “false-positives” represented significant decreases in LCMRglu (-8% to -32%). A number of “false-negative”

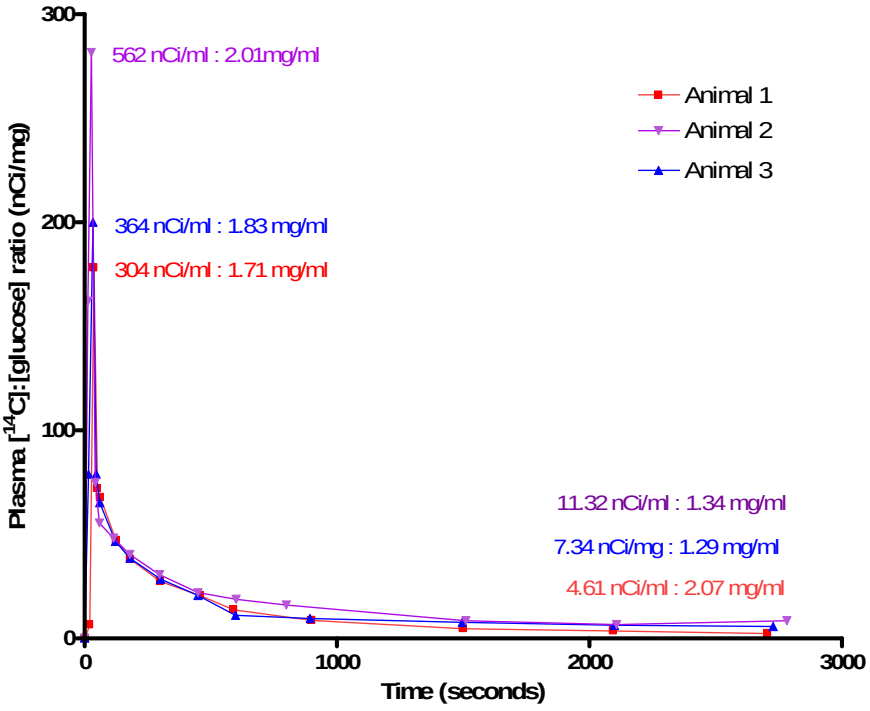
(3) results were also found when the corpus callosum was used as the selected reference region.

1.5 [^{14}C]-uptake ratio determination of LCMRglu

1.5.1 Terminal plasma ratio validity

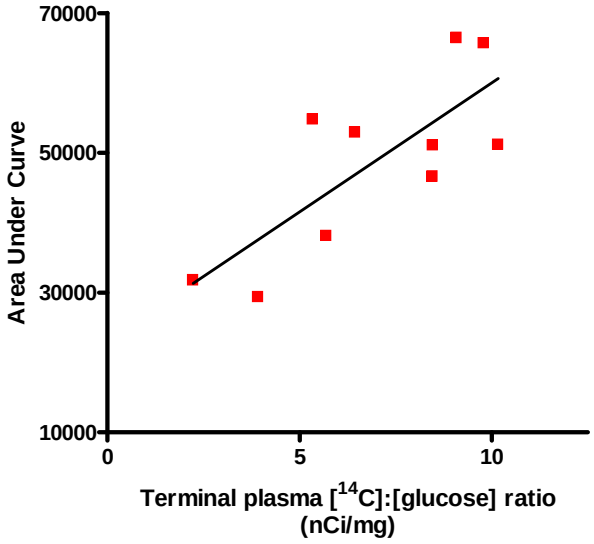
The area under the curve (integral) of the complete plasma history for each animal, as determined from the [^{14}C] / [glucose] ratio of 14 timed blood samples against the time at which the sample was taken, was significantly correlated with the [^{14}C] / [glucose] terminal plasma ratio (Figure 3.1.3).

Figure 3.1.2 Plasma history profile in quantitative 2-deoxyglucose autoradiography



Complete plasma history profile as determined in quantitative 2-deoxyglucose autoradiography. Data shown from 3 representative animals. The plasma [^{14}C] and [glucose] concentrations for each animal at the peak of isotope infusion and in the terminal plasma sample are also shown. Note in particular how the terminal plasma [^{14}C]:[glucose] ratio appears to reflect the peak amplitude of the plasma ratio history in each animal. This appears to be largely due to the fact that plasma [^{14}C]-2-DG concentration in the terminal plasma sample reflects that observed at the peak. This suggests that the terminal plasma [^{14}C] concentration, and plasma ratio, may provide an accurate reflection of that present at the peak.

Figure 3.1.3. Plasma history and terminal plasma-ratio relationship

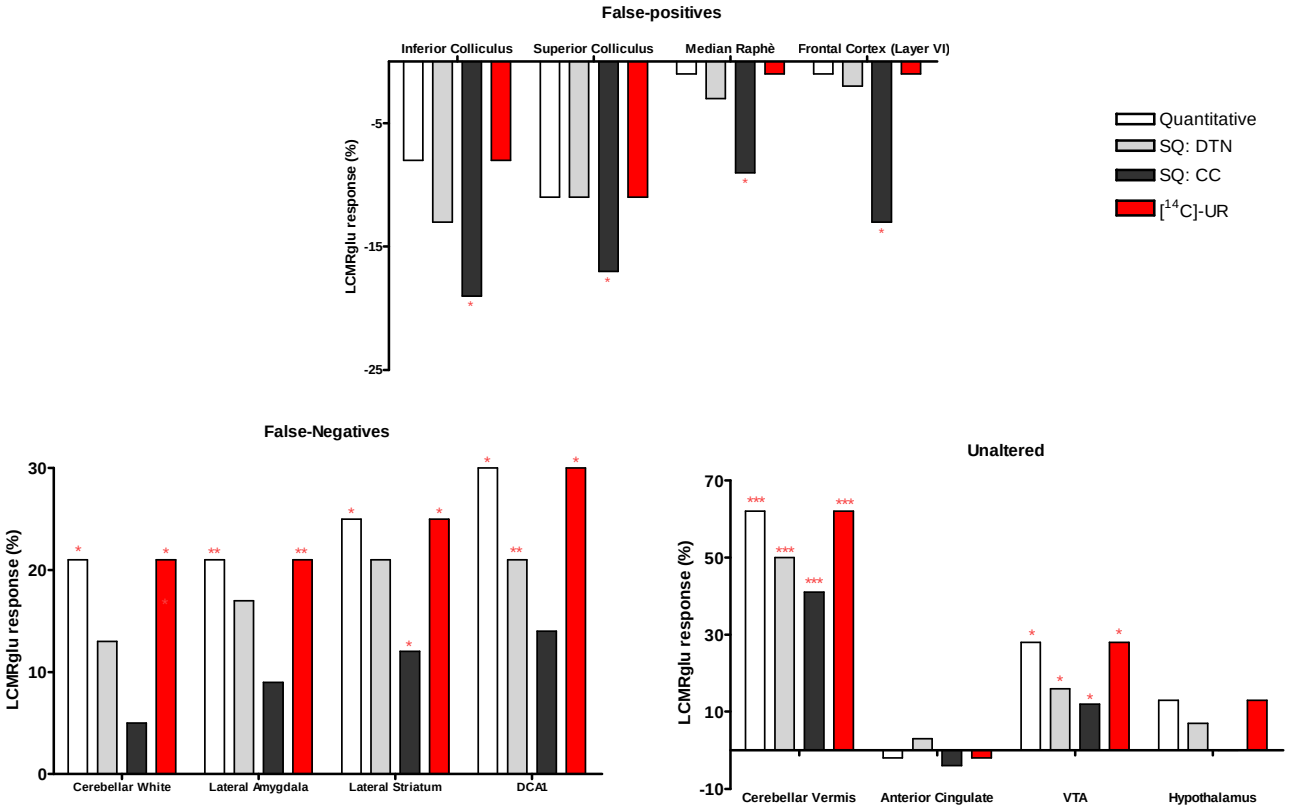


Correlation between complete plasma [^{14}C] / [glucose] history profile (Area under curve (integral)) and terminal plasma [^{14}C] / [glucose] ratio. (R^2=0.60, p=0.0087).The significant correlation between these parameters suggests that the terminal plasma [^{14}C]:[glucose] history provides an accurate reflection of the plasma history throughout the experimental time period.

LCMRglu

Changes in LCMRglu induced by MDMA and detected by [^{14}C]-UR analysis were similar in magnitude, directionality and localisation to those generated from quantitative analysis (see Figure 3.1.4 for representative examples). Of particular note was the fact that both MDMA-induced increases (in 21 of the 71 ROI) and decreases (in 1 ROI) were detected accurately by [^{14}C]-UR analysis with neither false negative nor false positive results being generated. The magnitude of the changes, increases ranging between range 20% to 81% and the decrease of -19%, were also similar to those found with the fully quantitative approach (Figure 3.1.4). Detailed data for all forms of analysis are shown in tables 3.1.2 to 3.1.7.

Figure 3.1.4 LCMRglu response to MDMA in quantitative, semi-quantitative and [^{14}C]-uptake ratio analysis



*LCMRglu responses to acute MDMA as detected by quantitative, semi-quantitative (SQ) and [^{14}C]-uptake ratio ([^{14}C]-UR) analysis. Data shown as % LCMRglu response in MDMA-treated animals in comparison to controls (saline-treated). Representative data shown from regions in which the significance of LCMRglu responses to MDMA is unaltered by the different analyses and regions in which false-negative and false-positive results are generated by SQ analysis. SQ:DTN represents data analysed with the dorsal tegmental nucleus and SQ:CC represents data analysed with the corpus callosum as the selected control region. *denotes p<0.05, **denotes p<0.01 and ***denotes p<0.001 significant difference from saline control (t-test). Note how the LCMRglu alterations detected by [^{14}C]-UR analysis are similar in magnitude, directionality and significance to those detected by quantitative analysis and that this form of analysis does not generate false-negative or false-positive results.*

Table 3.1.2 LCMRglu as determined by quantitative, semi-quantitative and [^{14}C]uptake ratio analysis: cortical regions

Cortical Brain Areas	Quantitative Analysis (µmol/100g/min)			Semi-quantitative analysis [^{14}C] ROI / [^{14}C] DTN			Semi-quantitative analysis [^{14}C] ROI / [^{14}C] Corpus Callosum			[^{14}C]-Uptake ratio analysis		
	Saline	**MDMA**	**%**	**Saline**	**MDMA**	**%**	**Saline**	**MDMA**	**%**	**Saline**	**MDMA**	**%**
Anterior Cingulate	119 ± 3	116 ± 9	**-2**	1.12 ± 0.07	1.15 ± 0.06	**3**	3.68 ± 0.19	3.55 ± 0.09	**-4**	15.07 ± 0.41	14.7 ± 1.13	**-2**
Frontal Layer IV	95 ± 3	103 ± 8	**9**	0.97 ± 0.05	1.05 ± 0.04	**9**	3.23 ± 0.13	3.08 ± 0.17	**-5**	12.02 ± 2.22	13.1 ± 0.96	**9**
Frontal Layer VI	87 ± 1	86 ± 5	**-1**	0.91 ± 0.03	0.90 ± 0.02	**-2**	3.02 ± 0.07	2.62 ± 0.08	**-13***	10.99 ± 0.51	10.87 ± 1.01	**-1**
Somatosensory Layer IV	114 ± 4	148 ± 7	**30****	1.15 ± 0.03	1.44 ± 0.06	**25****	3.79 ± 0.10	4.44 ± 0.12	**17****	14.46 ± 0.51	18.77 ± 0.86	**30****
Somatosensory Layer VI	83 ± 3	81 ± 7	**-3**	0.88 ± 0.01	0.84 ± 0.06	**-5**	2.91 ± 0.07	2.57 ± 0.06	**-12**	10.57 ± 0.20	10.29 ± 0.89	**-3**
Posterior Cingulate	117 ± 5	95 ± 8	**-19***	1.18 ± 0.05	0.96 ± 0.07	**-18***	3.88 ± 0.16	2.95 ± 0.06	**-24****	14.87 ± 0.61	12.05 ± 0.92	**-19***
Parietal Layer IV	94 ± 2	94 ± 8	**1**	0.95 ± 0.03	0.95 ± 0.06	**0**	3.18 ± 0.04	2.93 ± 0.07	**-8***	11.86 ± 0.28	11.92 ± 0.91	**1**
Parietal Layer VI	83 ± 1	83 ± 8	**0**	0.86 ± 0.03	0.84 ± 0.04	**-2**	2.87 ± 0.09	2.67 ± 0.08	**-7**	10.50 ± 0.11	10.50 ± 0.96	**0**
Piriform	54 ± 4	71 ± 1	**32**	0.61 ± 0.04	0.74 ± 0.04	**22**	2.01 ± 0.12	2.30 ± 0.13	**14**	6.80 ± 0.53	9.01 ± 0.97	**32**
Entorhinal	72 ± 3	81 ± 8	**12**	0.77 ± 0.03	0.84 ± 0.06	**8**	2.55 ± 0.08	2.58 ± 0.14	**1**	9.16 ±0.39	10.30 ± 1.01	**12**
Auditory	133 ± 5	150 ± 11	**13**	1.32 ± 0.66	1.43 ± 0.10	**11**	4.35 ± 0.19	4.50 ± 0.22	**4**	16.82 ± 0.67	19.03 ± 1.34	**13**
Occipital IV	109 ± 4	107 ± 10	**-2**	1.10 ± 0.04	1.07 ± 0.08	**-3**	3.65 ± 0.14	3.29 ± 0.10	**-10**	13.88 ± 0.54	13.62 ± 1.25	**-2**

*Local cerebral glucose utilisation (LCMRglu) measurements gained by quantitative, semi-quantitative and [^{14}C]-Uptakeratio analysis in cortical regions of MDMA-treated and control (saline-treated) rats. Semi-quantitative analysis completed with the corpus callosum and dorsal tegmental nucleus (DTN) as the selected control region. Data shown as mean ± s.e.m and % difference between MDMA-treated and control (saline-treated) animals.. *denotes p<0.05, **denotes p<0.01 significant difference from saline control (T-test).*

Table 3.1.3 LCMRglu as determined by quantitative, semi-quantitative and [^{14}C]uptake ratio analysis: motor regions

Motor Brain Areas	Quantitative Analysis (µmol/100g/min)			Semi-quantitative analysis [^{14}C] ROI / [^{14}C] DTN			[^{14}C] ROI / [^{14}C] Corpus Callosum			[^{14}C]-Uptake ratio analysis		
	Saline	MDMA	%	Saline	MDMA	%	Saline	MDMA	%	Saline	MDMA	%
Striatum												
Medial	93 ± 3	152 ± 10	**64*** **	0.96 ± 0.03	1.47 ± 0.09	**54*** **	3.16 ± 0.10	4.56 ± 0.21	**44*** **	11.77 ± 0.38	19.28 ± 1.24	**64*** **
Lateral	103 ± 4	129 ± 9	**25***	1.05 ± 0.03	1.27 ± 0.10	**21**	3.48 ± 0.12	3.90 ± 0.11	**12***	13.2 ± 0.49	16.39 ± 1.12	**25***
Ventrolateral Thalamus	92 ± 4	111 ± 10	**21**	0.95 ± 0.03	1.11 ± 0.06	**17**	3.13 ± 0.09	3.45 ± 0.23	**10**	11.64 ± 0.50	14.13 ± 1.21	**21**
Subthalamic Nucleus	80 ± 3	95 ± 10	**20**	0.84 ± 0.03	0.96 ± 0.06	**14**	2.77 ± 0.06	2.97 ± 0.19	**7**	10.10 ± 0.43	12.08 ± 1.33	**20**
Lateral Habenula	109 ± 10	87 ± 6	**-20**	1.09 ± 0.06	0.89 ± 0.08	**-18**	3.61 ± 0.25	2.75 ± 0.15	**-24***	13.80 ± 1.22	10.98 ± 0.79	**-20**
Substantia Nigra												
pars Reticulata	53 ± 3	96 ± 16	**81***	0.60 ± 0.02	0.95 ± 0.10	**59***	1.98 ± 0.05	2.96 ± 0.34	**50***	6.70 ± 0.35	12.13 ± 2.06	**81***
pars Compacta	64 ± 2	95 ± 8	**48****	0.70 ± 0.03	0.97 ± 0.09	**37***	2.33 ± 0.06	2.98 ± 0.21	**28***	8.17 ± 0.21	12.08 ± 0.98	**48****
Cerebellum												
Paramedian Lobule	52 ± 1	67 ± 5	**29***	0.59 ± 0.02	0.71 ± 0.04	**20***	1.96 ± 0.03	2.20 ± 0.06	**12****	6.57 ± 0.07	8.50 ± 0.59	**29***
Copula Pyramis	60 ± 2	96 ± 8	**61****	0.66 ± 0.02	0.97 ± 0.06	**47****	2.18 ± 0.05	2.99 ± 0.12	**37*** **	7.56 ± 0.20	12.20 ± 1.05	**61****
Vermis	85 ± 1	138 ± 9	**62*** **	0.89 ± 0.02	1.34 ± 0.05	**50*** **	2.94 ± 0.06	4.15 ± 0.14	**41*** **	10.81 ± 0.17	17.45 ± 1.09	**62*** **
Red Nucleus	70 ± 2	85 ± 7	**21**	0.75 ± 0.02	0.87 ± 0.05	**15**	2.49 ± 0.04	2.68 ± 0.12	**8**	8.90 ± 0.30	10.73 ± 0.93	**21**
Inferior Olive	79 ± 1	103 ± 6	**30****	0.83 ± 0.01	1.03 ± 0.06	**24***	2.76 ± 0.03	3.19 ± 0.08	**16****	10.00 ± 0.18	13.01 ± 0.78	**30****

*Local cerebral glucose utilisation (LCMRglu) measurements gained by quantitative, semi-quantitative and [^{14}C]-uptake ratio analysis in motor regions of MDMA-treated and control (saline-treated) Dark Agouti rats. Semi-quantitative analysis completed with the corpus callosum and dorsal tegmental nucleus (DTN) as the selected control region. Data shown as mean ± s.e.m and % difference between MDMA and control. *denotes p<0.05, **denotes p<0.01 significant difference from saline control (T-test).*

Table 3.1.4 LCMRglu as determined by quantitative, semi-quantitative and [^{14}C]uptake ratio analysis: limbic regions

Limbic Brain Areas	Quantitative Analysis (µmol/100g/min)			Semi-quantitative analysis						[^{14}C]-Uptake ratio analysis		
				[^{14}C] ROI / [^{14}C] DTN			[^{14}C] ROI / [^{14}C] Corpus Callosum					
	Saline	**MDMA**	**%**	**Saline**	**MDMA**	**%**	**Saline**	**MDMA**	**%**	**Saline**	**MDMA**	**%**
Nucleus Accumbens	87 ± 3	118 ± 7	**35****	0.91 ± 0.02	1.16 ± 0.05	**28****	3.00 ± 0.09	3.62 ± 0.19	**21***	11.06 ± 0.37	14.92 ± 0.86	**35****
Septal Nucleus	61 ± 4	83 ± 5	**35***	0.68 ± 0.04	0.85 ± 0.06	**25***	2.25 ± 0.14	2.63 ± 0.06	**17***	7.79 ± 0.49	10.48 ± 0.69	**35***
Anterior Thalamus	105 ± 5	93 ± 7	**-11**	1.06 ± 0.04	0.95 ± 0.08	**-11**	3.53 ± 0.19	2.92 ± 0.17	**-17***	13.29 ± 0.61	11.82 ± 0.88	**-11**
Mediodorsal Thalamus	102 ± 5	118 ± 9	**16**	1.04 ± 0.05	1.17 ± 0.06	**12**	3.44 ± 0.16	3.62 ± 0.14	**5**	12.96 ± 0.63	15.02 ± 1.16	**16**
Medial Amygdala	50 ± 5	68 ± 12	**37**	0.58 ± 0.06	0.71 ± 0.09	**23**	1.90 ± 0.15	2.16 ± 0.24	**14**	6.32 ± 0.58	8.63 ± 1.58	**37**
Lateral Amygdala	75 ± 2	91 ± 4	**21****	0.80 ± 0.03	0.93 ± 0.06	**17**	2.64 ± 0.10	2.86 ± 0.08	**9**	9.46 ± 0.31	11.49 ± 0.49	**21****
Ventral Tegmental Area	45 ± 2	57 ± 4	**28***	0.53 ± 0.01	0.61 ± 0.02	**16***	1.75 ± 0.04	1.96 ± 0.08	**12***	5.71 ± 0.26	7.28 ± 0.46	**28***
Mamillary Body	100 ± 4	104 ± 8	**3**	1.02 ± 0.02	1.04 ± 0.07	**2**	3.39 ± 0.09	3.21 ± 0.13	**-5**	12.71 ± 0.49	13.14 ± 1.03	**3**

*Local cerebral glucose utilisation (LCMRglu) measurements gained by quantitative, semi-quantitative and [^{14}C]-uptake ratio analysis in limbic brain regions of MDMA-treated and control (saline-treated) Dark Agouti rats. Semi-quantitative analysis completed with the corpus callosum and dorsal tegmental nucleus (DTN) as the selected control region. Data shown as mean ± s.e.m and % difference between MDMA and control. *denotes p<0.05, **denotes p<0.01 significant difference from saline control (T-test).*

Table 3.1.5 LCMRglu as determined by quantitative, semi-quantitative and [^{14}C]uptake ratio analysis: hippocampal regions

Hippocampal Regions	Quantitative Analysis (µmol/100g/min)			Semi-quantitative analysis						[^{14}C]-Uptake ratio analysis		
				[^{14}C] ROI / [^{14}C] DTN			[^{14}C] ROI / [^{14}C] Corpus Callosum					
	Saline	MDMA	%	Saline	MDMA	%	Saline	MDMA	%	Saline	MDMA	%
Molecular Layer	87 ± 8	94 ± 8	**9**	0.91 ± 0.08	0.96 ± 0.07	**6**	2.98 ± 0.23	2.96 ± 0.18	**-1**	11.04 ± 1.01	11.97 ± 0.97	**9**
Dorsal Subiculum	76 ± 5	84 ± 5	**9**	0.81 ± 0.05	0.87 ± 0.05	**6**	2.69 ± 0.17	2.68 ± 0.14	**0**	9.69 ± 0.59	10.60 ± 0.64	**9**
Dentate PO	56 ± 2	60 ± 6	**6**	0.63 ± 0.02	0.64 ± 0.05	**2**	2.08 ± 0.08	1.98 ± 0.09	**-5**	7.10 ± 0.26	7.56 ± 0.77	**6**
Dorsal CA1	58 ± 2	75 ± 6	**30***	0.64 ± 0.02	0.78 ± 0.03	**21****	2.13 ± 0.07	2.43 ± 0.13	**14**	7.31 ± 0.28	9.51 ± 0.82	**30***
CA2	56 ± 1	62 ± 4	**11**	0.63 ± 0.02	0.67 ± 0.04	**6**	2.09 ± 0.04	2.07 ± 0.07	**-1**	7.15 ± 0.16	7.91 ± 0.53	**11**
Ventral CA1	65 ± 3	74 ± 4	**15**	0.7 ± 0.0.3	0.78 ± 0.05	**10**	2.32 ± 0.07	2.39 ± 0.07	**3**	8.19 ± 0.35	9.39 ± 0.52	**15**
Ventral Subiculum	64 ± 5	67 ± 6	**5**	0.70 ± 0.05	0.72 ± 0.05	**2**	2.32 ± 0.14	2.21 ± 0.09	**-5**	8.14 ± 0.62	8.55 ± 0.81	**5**
CA3	67 ± 2	66 ± 6	**-1**	0.73 ± 0.01	0.71 ± 0.06	**3**	2.40 ± 0.04	2.17 ± 0.11	**-10**	8.50 ± 0.26	8.40 ± 0.75	**-1**

*Local cerebral glucose utilisation (LCMRglu) measurements gained by quantitative, semi-quantitative and [^{14}C]-uptake ratio analysis in hippocampal regions of MDMA-treated and control (saline-treated) Dark Agouti rats. Semi-quantitative analysis completed with the corpus callosum and dorsal tegmental nucleus (DTN) as the selected control region. Data shown as mean ± s.e.m and % difference between MDMA and control. *denotes p<0.05, **denotes p<0.01 significant difference from saline control (T-test).*

Table 3.1.4 LCMRglu as determined by quantitative, semi-quantitative and [^{14}C]uptake ratio analysis: sensory regions

Sensory Brain Regions	Quantitative Analysis (µmol/100g/min)			Semi-quantitative analysis [^{14}C] ROI / [^{14}C] DTN			[^{14}C] ROI / [^{14}C] Corpus Callosum			[^{14}C]-Uptake ratio analysis		
	Saline	**MDMA**	**%**	**Saline**	**MDMA**	**%**	**Saline**	**MDMA**	**%**	**Saline**	**MDMA**	**%**
Superior Olive	113 ± 4	109 ± 8	**-3**	1.13 ± 0.02	1.09 ± 0.06	**-4**	3.74 ± 0.10	3.37 ± 0.13	**-10**	14.28 ± 0.54	13.83 ± 0.96	**-3**
Trigeminal Nucleus	61 ± 1	84 ± 8	**39***	0.67 ± 0.02	0.85 ± 0.05	**27****	2.22 ± 0.04	2.63 ± 0.10	**19****	7.71 ± 0.18	10.71 ± 0.97	**39***
Lateral Geniculate	92 ± 3	89 ± 10	**-3**	0.95 ± 0.02	0.90 ± 0.06	**-5**	3.14 ± 0.10	2.79 ± 0.15	**-11**	11.62 ± 0.44	11.31 ± 1.29	**-3**
Superior Colliculus												
Superficial Layers	109 ± 6	97 ± 10	**-11**	1.10 ± 0.05	0.98 ± 0.06	**-11**	3.64 ± 0.13	3.03 ± 0.16	**-17***	13.88 ± 0.59	12.33 ± 1.27	**-11**
Deep Layers	86 ± 3	102 ± 9	**19**	0.90 ± 0.04	1.03 ± 0.07	**15**	2.96 ± 0.11	3.17 ± 0.15	**7**	10.88 ± 0.35	12.99 ± 1.17	**19**
Inferior Colliculus	142 ± 5	131 ± 10	**-8**	1.40 ± 0.05	1.23 ± 0.13	**-13**	4.65 ± 0.20	3.74 ± 0.27	**-19***	18.06 ± 0.66	16.62 ± 1.33	**-8**

*Local cerebral glucose utilisation (LCMRglu) measurements gained by quantitative, semi-quantitative and [^{14}C]-uptake ratio analysis in sensory regions of MDMA-treated and control (saline-treated) Dark Agouti rats. Semi-quantitative analysis completed with the corpus callosum and dorsal tegmental nucleus (DTN) as the selected control region. Data shown as mean ± s.e.m and % difference between MDMA and control. *denotes p<0.05, **denotes p<0.01 significant difference from saline control (T-test).*

Table 3.1.7 LCMRglu as determined by quantitative, semi-quantitative and [^{14}C]uptake ratio analysis: non-specific regions

Non-specific Brain Regions	Quantitative Analysis (µmol/100g/min)			Semi-quantitative analysis						[^{14}C]-Uptake ratio analysis		
				[^{14}C] ROI / [^{14}C] DTN			[^{14}C] ROI / [^{14}C] Corpus Callosum					
	Saline	MDMA	%	Saline	MDMA	%	Saline	MDMA	%	Saline	MDMA	%
Nucleus Gelatinosus	110 ± 3	117 ± 9	**6**	1.12 ± 0.05	1.16 ± 0.08	**4**	3.69 ± 0.14	3.59 ± 0.15	**-3**	13.98 ± 0.44	14.87 ± 1.17	**6**
Cerebellar White	25 ± 1	30 ± 2	**21***	0.33 ± 0.01	0.37 ± 0.03	**13**	1.08 ± 0.03	1.13 ± 0.04	**5**	3.17 ± 0.16	3.83 ± 0.22	**21***
Corpus Callosum	22 ± 1	26 ± 2	**19***	0.30 ± 0.01	0.33 ± 0.02	**7**	n/a	n/a	**n/a**	2.74 ± 0.10	3.27 ± 0.20	**19***
Nucleus Reunines	83 ± 6	95 ± 12	**15**	0.87 ± 0.04	0.95 ± 0.06	**10**	2.87 ± 0.17	2.99 ± 0.33	**4**	10.53 ± 0.82	12.08 ± 1.49	**15**
Medial Habenula	78 ± 3	75 ± 4	**-4**	0.83 ± 0.04	0.78 ± 0.04	**-6**	2.74 ± 0.11	2.41 ± 0.06	**-12***	9.89 ± 0.37	9.46 ± 0.50	**-4**
Hypothalamus	54 ± 2	60 ± 4	**13**	0.61 ± 0.02	0.65 ± 0.03	**7**	2.01 ± 0.07	2.01 ± 0.04	**0**	6.80 ± 0.30	7.66 ± 0.53	**13**
Internal capsule	26 ± 1	34 ± 3	**31**	0.34 ± 0.01	0.40 ± 0.03	**18**	1.11 ± 0.02	1.23 ± 0.05	**10**	3.30 ± 0.12	4.31 ± 0.43	**31**
Pontine Reticular Formation	53 ± 1	62 ± 4	**17**	0.6 ± 0.02	0.67 ± 0.04	**11**	1.99 ± 0.05	2.07 ± 0.09	**4**	6.72 ± 0.16	7.84 ± 0.52	**17**
Dorsal Raphe	81 ± 1	85 ± 6	**4**	0.86 ± 0.02	0.87 ± 0.05	**2**	2.83 ± 0.06	2.69 ± 0.09	**-5**	10.32 ± 0.17	10.76 ± 0.80	**4**
Median Raphe	90 ± 2	89 ± 7	**-1**	0.93 ± 0.02	0.09 ± 0.05	**-3**	3.08 ± 0.06	2.79 ± 0.10	**-9***	11.39 ± 0.22	11.26 ± 0.92	**-1**
Dorsal Tegmental Nucleus	98 ± 3	93 ± 7	**-5**	n/a	n/a	**n/a**	3.31 ± 0.06	3.13 ± 0.23	**-5**	12.41 ± 0.43	11.80 ± 0.93	**-5**
Nucleus Ambiguus	62 ± 1	86 ± 5	**40****	0.68 ± 0.02	0.89 ± 0.04	**30****	2.26 ± 0.04	2.75 ± 0.11	**22****	7.84 ± 0.13	10.96 ± 0.58	**40****

*Local cerebral glucose utilisation (LCMRglu) measurements gained by quantitative, semi-quantitative and [^{14}C]-uptake ratio analysis in non-specific regions of MDMA-treated and control (saline-treated) Dark Agouti rats. Semi-quantitative analysis completed with the corpus callosum and dorsal tegmental nucleus (DTN) as the selected control region. Data shown as mean ± s.e.m and % difference between MDMA and control. *denotes p<0.05, **denotes p<0.01 significant difference from saline control (T-test). n/a denotes measurements not available due to form of analysis being applied.*

1.6 Discussion and Conclusions

These results suggest that the SQ analytical method as used previously suffers from reduced accuracy and reduced validity in comparison to the fully quantitative method. The inaccuracies are found in the magnitude, directionality and localisation of drug-induced changes in LCMRglu and the data are markedly affected by the choice of reference area. The DTN meets the criterion for choice as a reference region as LCMRglu was not significantly altered by MDMA treatment when analysed using quantitative analysis. However, using this reference region resulted in a number of "false-negative" results being generated, suggesting that this method has a reduced statistical power in comparison to the quantitative approach. While it might be possible to increase statistical power by the simple expedient of increasing sample size, it would appear that it cannot be solved by more judicious choice of reference region. A more extensive analysis (data not shown) in which a number of other areas of the brain were used as the reference region, on the basis that they both fulfilled the required criteria as an appropriate reference region by the SQ method and were also confirmed to remain unaltered across treatment groups by fully quantitative analysis, it was found that most also resulted in the generation of "false-positive" results. This finding, along with the observation that the methods employed in SQ analysis are not sufficiently robust to appropriately establish if the rate of LCMRglu is constant within a selected reference region between experimental groups, considerably weakens the validity of this analytical technique. Indeed, we have demonstrated that when a region in which a significant alteration in LCMRglu is not detected by the methods used in SQ analysis is used as the selected reference region (as exemplified by the corpus callosum in this study) the weaknesses of this analytical technique are further compounded. In addition to these problems the requirement for consistent plasma parameters and the use of a predetermined reference region in SQ analysis also limit the experimental conditions in which the method can be applied, as many experimental manipulations result in altered plasma glucose and/ or global alterations in LCMRglu.

The development of the alternative analytical approach to the SQ 2-DG methodology, as outlined here, presents several advantages over the approaches used previously. By approximating as closely as possible to the original operational equation, there is no need to use a reference brain region against which to standardise the regions of interest thus avoiding the potentially problematic assumption of stability of this region across treatment groups. As a direct consequence of this, our novel approach can be used in a wider variety of

experimental conditions where either altered plasma parameters or when global alterations in LCMRglu occur. Additionally, our analyses have confirmed that the terminal plasma [^{14}C] / [glucose] ratio provides a close approximation to the plasma history profile of these parameters within each animal allowing it to be used with a fair degree of certainty in the determination of LCMRglu by [^{14}C]-UR analysis. However, it is important to note that for valid determination of LCMRglu with [^{14}C]-UR analysis several assumptions are accepted that are exactly equivalent to those incorporated in the quantitative technique and it is worth considering whether these assumption are at all compromised by our novel analytical method.

A principal assumption of our novel approach is that the relationship between the rate of [^{14}C]-2-DG incorporation into the cerebral tissue and the plasma [^{14}C] / [glucose] history is consistent between experimental groups. This assumption provides the foundation for adjustment of the initial ratio in [^{14}C]-UR analysis to accommodate individual animal and group plasma ratio variability, and directly parallels the assumption in the fully quantitative approach that the rate constants for carrier mediated transport and phosphorylation of [^{14}C]-2-deoxyglucose, and the lumped constant, are consistent between different experimental groups. However, it is accepted that the certainty of this assumption is likely to diminish if the glycaemic state of animals within the different experimental groups is vastly different. Indeed, both the kinetic and lumped constants have been shown to alter under conditions of severe hyperglycaemia (Schuier et al., 1981; Sokoloff et al., 1977). The requirement that all animals are in the normoglycaemic range for the fully quantitative approach to 2-deoxyglucose imaging is likely to be even more important for our novel approach. Indeed, it is also possible the SQ approach is actually more sensitive to violations of these assumptions arising from altered 2-DG kinetics than is the fully quantitative approach. In quantitative analysis the contribution of these kinetic values to the operational equation is minimised by the relatively long experimental time, by the end of which only low levels of plasma [^{14}C]-2-DG are present relative to the large peak of plasma [^{14}C]-2-DG a few seconds after *i.v* bolus injection. However, when injected *i.p* the clearance of [^{14}C]-2-DG from the plasma is delayed and higher residual levels are present at the experimental end-point (Kelly and McCulloch, 1983a; Meibach et al., 1980) and in terms of impact upon the operational equation, the contribution of the factors containing the kinetic constants will be increased and any error that results from altered [^{14}C]-2-DG kinetics will be enhanced. The observation that unphopshorylated levels of [^{14}C]-2-DG in the CNS are increased following *i.p* injection give some credence to this suggestion. Although some of the uncertainty can be eliminated by ensuring that there are no inter-group differences in glycaemic state that will introduce

systematic errors into the determination of LCMRglu, the lack of a timed sampling protocol to accurately define plasma histories of glucose and tracer in the SQ methodology adds the risk of missing any transient alterations in glycaemic state that may interfere with valid LCMRglu estimation.

In many experiments using a modified SQ [^{14}C]-2-DG technique it is assumed that the levels of tracer measured in the brain represent phosphorylated 2-DG with levels of unphosphorylated [^{14}C]-2-DG so close to zero that they can be ignored. Indeed, we have made this very assumption in the derivation from the original quantitative operational equation of the initial ratio for [^{14}C]-UR analysis. It has previously been demonstrated, however, that under some experimental conditions a significant proportion of the total [^{14}C] present is in the form of unphosphorylated [^{14}C]-2-DG. Such conditions include the injection of tracer by *i.p.* rather than *i.v.* routes, when a short (30 minute) experimental time period is used, or when animals are hyperglycaemic (Kelly and McCulloch, 1983a). These problems can be largely reduced by the use of a longer (45 minute) experimental time period and by the intrinsic properties inherent in the [^{14}C]-UR analytical method itself. In addition, if similar glycaemic states (within the normoglycaemic range) are maintained as a prerequisite in the experimental design then the influence of this factor on LCMRglu determination is limited. Furthermore, as LCMRglu is estimated in one experimental group in direct comparison to the control group and adjustment is made to the initial ratio in [^{14}C]-UR analysis to incorporate the plasma variables for each animal, any potential influence of slight variability in the glycaemic state of the animals is controlled for. In order to circumvent the possible influence of increased unphosphorylated [^{14}C]-2-DG the brain following *i.*p injection and variation in glycaemic state on LCMRglu determination, brain and plasma samples were routinely collected 45 minutes after [^{14}C]-2-DG injection and the glycaemic state of animals in all groups was ensured to be within the normoglycaemic range in all experiments completed in this thesis.

In conclusion, the novel analytical approach to the SQ 2-DG approach outlined here represents an increase in accuracy that brings data very close to that generated by the fully quantitative technique. Any loss in accuracy from the fully quantitative approach is offset by the wider experimental design that can be accommodated. The [^{14}C]-UR analytical method was used in the determination of LCMRglu in all of the [^{14}C]-2-DG autoradiographic imaging studies completed withing this thesis.

2. Study 2 - Constitutive brain function in *h*SERT over-expressing mice

2.1 Rationale

SERT mRNA levels have been shown to be enhanced in the raphé nuclei of *h*SERT OVR mice (Jennings et al., 2006) supporting the contention that SERT gene transcription is enhanced in the raphè 5-HT neurones of these animals. Furthermore, this enhanced SERT gene transcription results in an increased level of SERT protein expression, as detected by [^{3}H]paroxetine binding, in membranes from the frontal cortex and brainstem for these animals (Loder et al., 2000). However, the possibility that the increased SERT expression present in the CNS of *h*SERT OVR mice may be regionally heterogenous has not been addressed. As such heterogeneity may be central to the effects of increase SERT gene transcription on affective, cerebral and serotonin system functioning here we use [^{3}H]paroxetine binding autoradiography to characterise SERT protein expression in 46 discrete brain regions in *h*SERT OVR mice in comparison to their Wt littermates.

In humans the influence of the 5-HTTLPR on affective functioning is greater in females than in males. Gender-dependent modulation of the increase in SERT protein expression that results from the increased SERT transcription in "long" allele individuals of this polymorphisms may represent a primary mechanism by which gender influences the effect of the 5-HTTLPR on affective functioning. To assess the possible influence of gender on a genetically driven increase in SERT expression here we investigate [^{3}H]paroxetine binding in both male and female *h*SERT OVR and Wt mice. In parallel to the known gender influence of the 5-HTTLPR on affective functioning we hypothesise that the magnitude of increased SERT expression that results from the enhanced SERT gene transcription in *h*SERT OVR mice will be greater in females than in males.

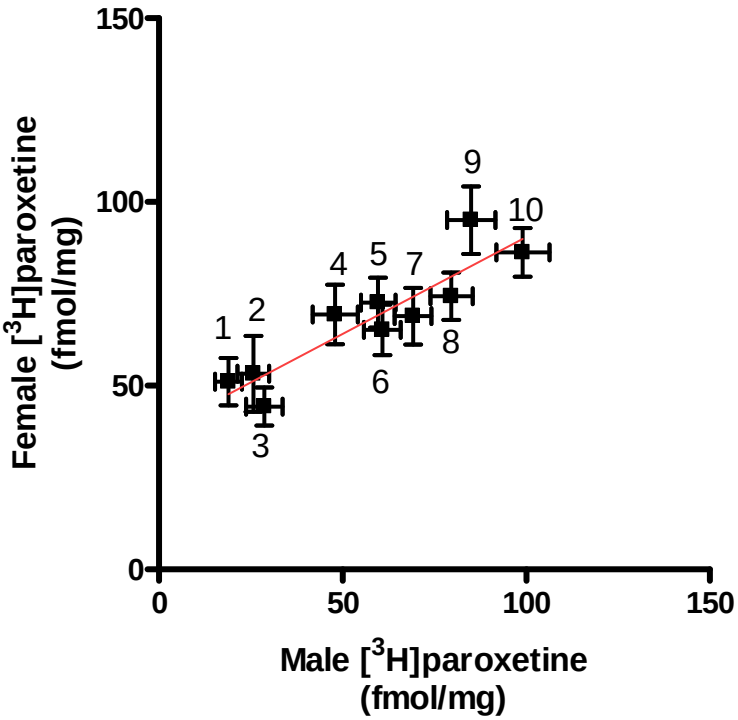
As a life-long increase in SERT function results in reduced *trait* anxiety in humans (Lesch et al., 1996), and reduced anxiety-like behaviour under normal behavioural conditions in *h*SERT OVR mice (Jennings et al., 2006), alterations in constitutive cerebral functioning are likely. Here we use the SQ [^{14}C]-2-DG to determine LCMRglu in *h*SERT OVR and Wt mice to investigate the effect of a life-long increase in SERT expression on constitutive cerebral function. Increased SERT protein expression present in *h*SERT OVR mice has been shown to result in decreased extracellular 5-HT levels in the CNS (Jennings et al., 2006).

The relationship between extracellular 5-HT availability and cerebral metabolism appears to be brain-region dependent. In most brain regions an acute enhancement of 5-HT neurotransmission is associated with a decrease in cerebral glucose metabolism (McBean et al., 1999; Smith et al., 2002a; Smith et al., 2002b). However, results from other studies in which 5-HT neurotransmission is pharmacologically enhanced or depleted suggest that 5-HT stimulates cerebral metabolism some brain regions, including the orbitofrontal and prefrontal cortex (Bremner et al., 1997; Mann et al., 1996). Furthermore, electrical stimulation of raphé 5-HT neurones has been found to increase cerebral metabolism in a number of other brain regions, including the thalamus, mamillary body and components of the basal ganglia (Cudennec et al., 1988b). Therefore, we hypothesise that an increase in constitutive cerebral metabolism is likely to be present in the majority of brain regions in *h*SERT OVR mice with localised decreases observed in a limited number of brain regions as a result of the decreased synaptic 5-HT levels present in these animals. Furthermore, we hypothesis that in parallel with the known influence of gender on the effects of a life-long increase in SERT function on affective functioning, constitutive cerebral metabolism with be altered to greater extent in female *h*SERT OVR mice than in males.

2.2 Serotonin transporter binding

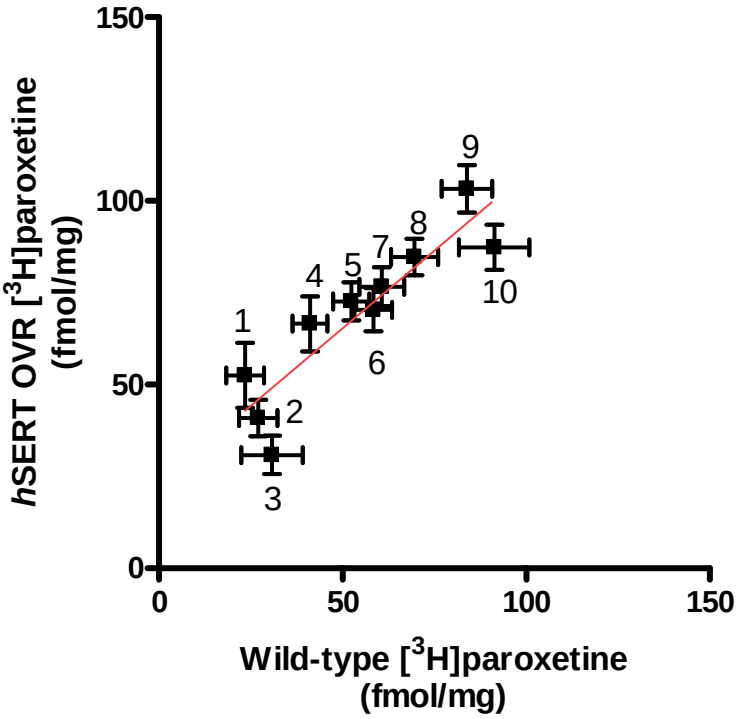
The distribution and concentration of [^{3}H]paroxetine binding in this study was similar to that reported by others (DeSouza and Kuyatt, 1987; Reader et al., 1998; Sharkey et al., 1991). The regional pattern of [^{3}H]paroxetine binding sites was similar between both genders and genotypes. There was a strong correlation between all binding data in male and female mice ($r=0.734$, $p<0.0001$) and Wt and *h*SERT OVR mice ($r=0.837$, $p<0.0001$) indicative of a similar SERT expression profile across the genders and genotypes. Figures 3.2.1 and 3.2.2 demonstrate the correlative nature of [^{3}H]paroxetine binding between genders and genotypes, respectively, as shown in 10 representative brain regions. In all animals [^{3}H]paroxetine binding was highest in the dorsal and median raphé, the molecular layer of the hippocampus, amygdala nuclei, hypothalamus and the substantia nigra. Medium levels of binding were evident in subregions of the hippocampus and the nucleus accumbens whereas low levels of binding were detected in the striatum and anterior cortical regions (e.g. mPFC, anterior cingulate and frontal cortex).

Figure 3.2.1 Regional correlation of [^{3}H]paroxetine binding between male and female mice



Regional correlation of [^{3}H]paroxetine binding in male and female mice. Data shown for 10 selected representative brain regions. 1= ventrolateral thalamus, 2= posterior cingulate, 3= dentate PO, 4= anterior cingulate, 5= CA3, 6=orbitofrontal cortex, 7= dorsal subiculum, 8= frontal cortex, 9= basolateral amygdala, 10= dorsal raphé. Data from both Wt and hSERT OVR mice were included in this analysis which showed a strong and significant correlation (r= 0.908, p<0.0001).This suggests that the regional distribution of SERT expression is similar between male and female mice.

Figure 3.2.2 Regional correlation of [^{3}H]paroxetine binding in *h*SERT OVR and Wt mice

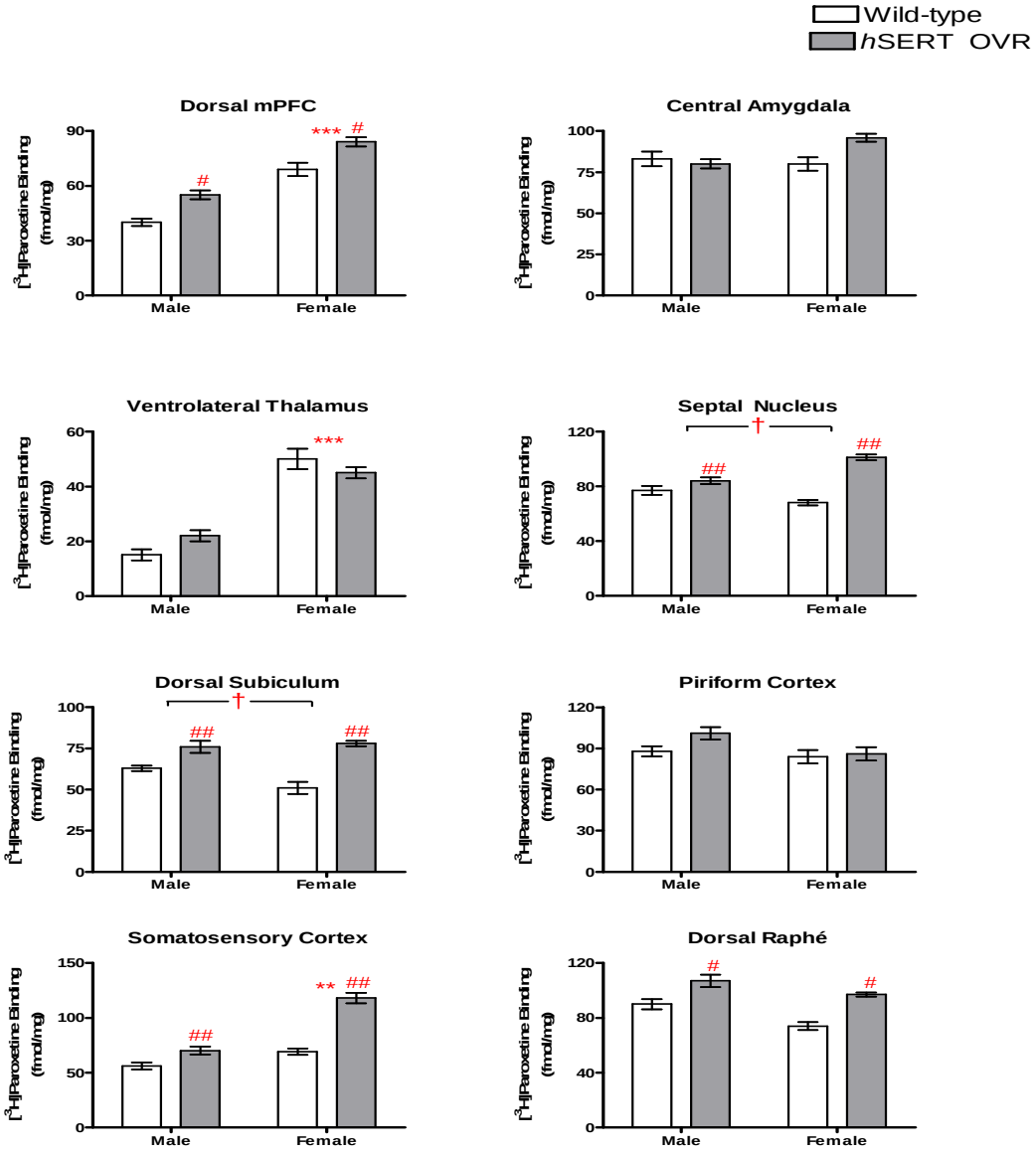


Regional correlation of [^{3}H]paroxetine binding in Wt and hSERT OVR animals. Data shown for 10 representative brain regions. 1= posterior cingulate, 2= dentate PO, 3= ventrolateral thalamus, 4= anterior cingulate, 5= orbitofrontal cortex, 6= CA3, 7= dorsal subiculum, 8= frontal cortex, 9= dorsal raphé, 10= basolateral amygdala. Data from both male and female mice were included in this representative analysis which showed a strong and significant correlation (r= 0.9, p<0.0001). This suggests that the regional expression of SERT is similar between hSERT OVR and Wt mice.

In the majority of brain regions [^{3}H]paroxetine binding tended to be greater in females than in males. Indeed, [^{3}H]paroxetine binding was found to be significantly higher in females as compared to males in a 18 of the 42 brain regions analysed. This effect was particularly prevalent in cortical regions (including the dorsal mPFC +65%, posterior cingulate +97% and somatosensory cortex +46%), the hippocampus (including the dentate PO +53%, CA2 +30%, ventral CA1 +51%), components of the basal ganglia (medial striatum +27%, globus pallidus +70%) and thalamic regions (mediodorsal +47%, ventrolateral +173%) (Figure 3.2.3). In contrast, there was no evidence for a significant gender difference in [^{3}H]paroxetine binding in any of the amygdala or raphé nuclei. See Figures 3.2.3 and 3.2.4

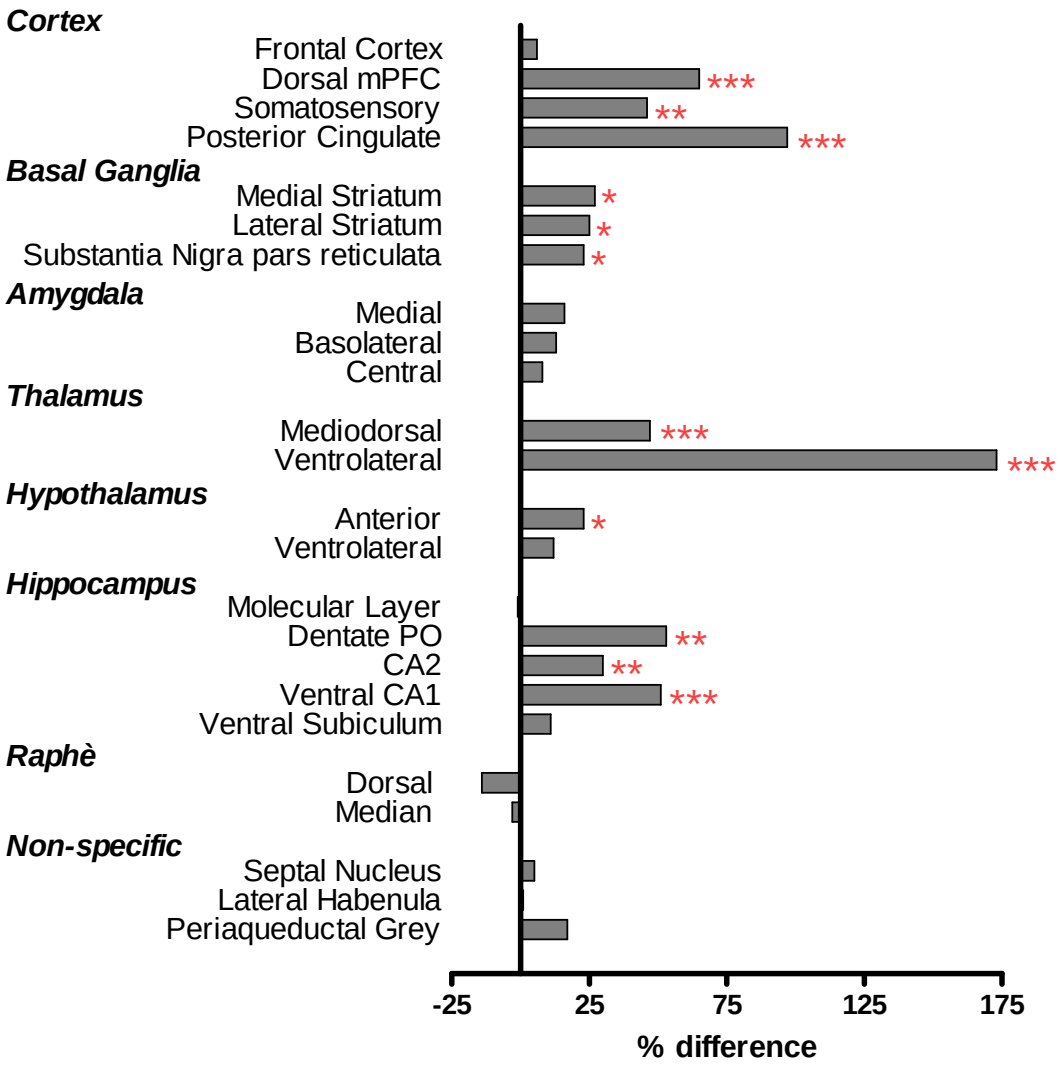
for representative regions in which gender influences [^{3}H]paroxetine binding. Detailed [^{3}H]paroxetine binding data are shown in Tables 3.2.1 to 3.2.5.

Figure 3.2.3 Effect of gender and *h*SERT over-expression on [^{3}H]Paroxetine binding



*[^{3}H]Paroxetine binding (fmol/mg) in 8 representative brain regions of wild-type and hSERT OVR male and female mice. Data were analysed using 2-WAY ANOVA. **denotes p<0.01 and ***denotes p<0.001 significant gender effect. $^{\#}$denotes p<0.05 and $^{\#\#}$denotes p<0.01 significant genotype effect. †denotes p<0.05 significant gender x genotype interaction. Note in particular the increased [^{3}H]paroxetine binding in some brain regions of females as compared to males (e.g. Dorsal mPFC) and how hSERT over-expression results in increased binding in some brain regions (e.g. somatosensory cortex) but not others (e.g. central amygdala). Furthermore, note how the increased [^{3}H]paroxetine binding induced by hSERT over-expression is greater in females than in males in some brain regions (e.g. septal nucleus)*

Figure 3.2.4 Gender differences in [^{3}H]paroxetine binding

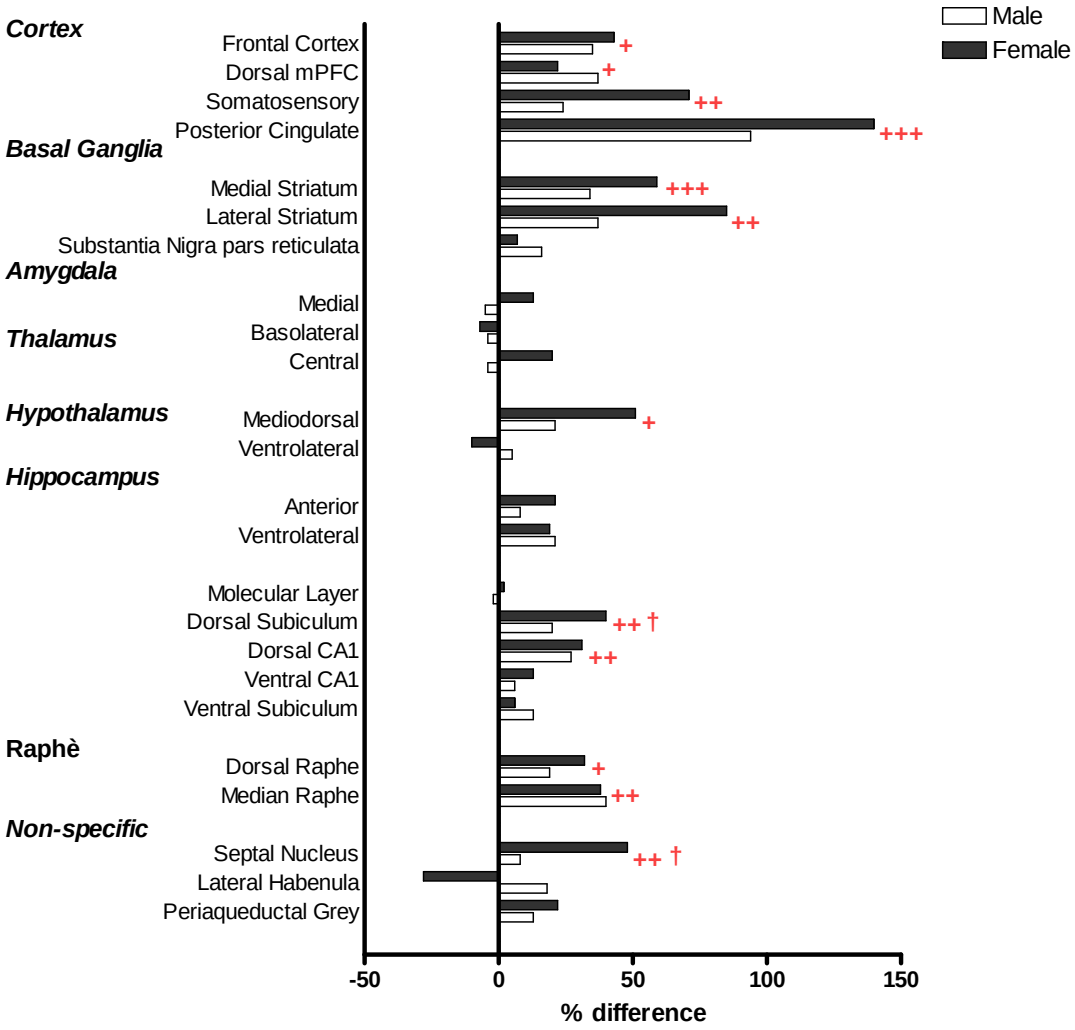


*Gender differences in [^{3}H]paroxetine binding in representative brain regions of diverse functional systems. Data shown as % difference of binding in female animals as compared to males, with data gained from both Wt and hSERT OVR animals. *denotes p<0.05, **denotes p<0.01, ***denotes p<0.001 significant gender difference (2-way ANOVA). Gender differences in some regions appear to be extremely large when shown as a % due to the relatively low level of actual [^{3}H]paroxetine binding present in these regions (e.g. ventrolateral thalamus [^{3}H]paroxetine male:26 fmol/mg female:47 fmol/mg).*

In both male and female animals the density of [^{3}H]paroxetine binding tended to be greater in *h*SERT OVR mice compared to Wt animals in the majority of brain areas. Indeed, [^{3}H]paroxetine binding was found to be significantly increased in *h*SERT OVR mice in 20 of the 42 brain regions analysed. These significant increases were present in cortical regions (range +34% to 59%), multiple hippocampal subfields (range +30 to +36%), components of the basal ganglia (range +38% to +42%), thalamic regions (range +25% to 38%) and the raphé nuclei (range 38% to 39%). However, [^{3}H]paroxetine binding was not significantly altered in *h*SERT OVR animals in a number of brain regions including the piriform cortex (+8%) or any of the amygdala nuclei (range -5% to +8%) indicating that increased SERT expression in *h*SERT OVR mice is regionally heterogenous. See Figures 3.2.3 and 3.2.5 for representative examples of how *h*SERT over-expression alters [^{3}H]paroxetine binding in a brain region dependent manner. Detailed data on [^{3}H]paroxetine binding in Wt and *h*SERT OVR mice are shown in Tables 3.2.1 to 3.2.5.

In the majority of brain regions the magnitude of the increase in [^{3}H]paroxetine binding present in *h*SERT OVR animals as compared to Wt animals tended to be greater in females than in males (see Figure 3.2.5). This suggests that gender may modulate the ability of *h*SERT OVR to increase SERT protein expression levels and this was confirmed by the presence of a significant gender x genotype interaction in two brain regions, the septal nucleus (+40% greater increase in females) and the dorsal subiculum (+34% greater increase in females).

Figure 3.2.5 Effect of *h*SERT over-expression of [^{3}H]paroxetine binding



Effect of hSERT over-expression on [^{3}H]paroxetine binding in selected brain regions. Data shown as % difference in binding of hSERT OVR mice in comparison to Wt mice of the same gender. $^{+}$denotes p<0.05, $^{++}$denotes p<0.01 and $^{+++}$denotes p<0.001 significant genotype effect. †denotes p<0.05 significant gender x genotype interaction. Data were analysed using 2-way ANOVA. Note how the presence of the hSERT transgene results in significantly increased [^{3}H]paroxetine binding in some brain regions (e.g. cortical regions) but not others (e.g. amygdale). Furthermore, note how the magnitude of the increase in [^{3}H]paroxetine binding as a result of hSERT over-expression is greater in females than in males in a limited number of brian regions (dorsal subiculum and septal nucleus).

Table 3.2.1 [^{3}H]Paroxetine binding in *h*SERT OVR and Wt mice: cortical regions

	Male							Female						
	Wild-type			*h*SERT OVR			%	Wild-type			*h*SERT OVR			%
	Mean		s.e.m	Mean		s.e.m	difference	Mean		s.e.m	Mean		s.e.m	difference
Cortex														
Orbitofrontal	52	±	7	70	±	6	**35**$^{###}$	53	±	5	76	±	6	**43**$^{###}$
Frontal	72	±	9	87	±	6	**21**$^{#}$	66	±	6	82	±	6	**23**$^{#}$
Anterior Cingulate	38	±	6	57	±	9	**52**$^{###}$	50*	±	6	81*	±	7	**60**$^{###}$
Dorsal medial Prefrontal	40	±	5	55	±	6	**37**$^{#}$	69***	±	9	84***	±	6	**22**$^{#}$
Ventral medial Prefrontal	45	±	5	68	±	5	**50**$^{##}$	73***	±	5	83***	±	8	**13**$^{##}$
Somatosensory	56	±	8	70	±	9	**24**$^{##}$	69**	±	7	118**	±	12	**71**$^{##}$
Temperoparietal	30	±	8	59	±	7	**99**$^{##}$	53	±	6	78	±	7	**45**$^{##}$
Posterior Cingulate	18	±	5	34	±	5	**94**$^{###}$	31***	±	6	75***	±	9	**140**$^{###}$
Piriform	88	±	9	101	±	11	**14**	84	±	12	86	±	12	**2**
Entorhinal	87	±	9	88	±	10	**2**	72	±	4	96	±	8	**33**

*Effect of hSERT over-expression on [^{3}H]Paroxetine binding in cortical and basal ganglia regions. Data are expressed as mean ± s.e.m and were analysed using 2-way ANOVA. *denotes p<0.05, **denotes p<0.01, ***denotes p<0.001 significant gender difference. $^{#}$denotes p<0.05, $^{##}$denotes p<0.01 and $^{###}$denotes p<0.001 significant genotype effect.*

Table 3.2.2 [^{3}H]Paroxetine binding in *h*SERT OVR and Wt mice: basal ganglia

	Male							Female						
	Wild-type			*h*SERT OVR			%	Wild-type			*h*SERT OVR			%
	Mean		s.e.m	Mean		s.e.m	difference	Mean		s.e.m	Mean		s.e.m	difference
Basal Ganglia														
Medial Striatum	47	±	6	63	±	6	**34**$^{###}$	55*	±	6	87*	±	9	**59**$^{###}$
Lateral Striatum	34	±	7	47	±	9	**37**$^{##}$	36*	±	5	68*	±	6	**85**$^{##}$
Globus Pallidus	35	±	6	58	±	7	**66**$^{#}$	74***	±	4	86***	±	9	**17**$^{#}$
Subthalamic Nucleus	42	±	6	45	±	9	**7**	37	±	5	32	±	5	**-13**
Substantia Nigra pars Reticulata	88	±	5	102	±	9	**16**	113*	±	11	120*	±	12	**7**
Substantia Nigra pars Compacta	74	±	6	91	±	7	**22**	90	±	4	113	±	12	**25**

*Effect of hSERT over-expression on [^{3}H]Paroxetine binding in cortical and basal ganglia regions. Data are expressed as mean ± s.e.m and were analysed using 2-way ANOVA. *denotes p<0.05, **denotes p<0.01, ***denotes p<0.001 significant gender difference. $^{#}$denotes p<0.05, $^{##}$denotes p<0.01 and $^{###}$denotes p<0.001 significant genotype effect.*

Table 3.2.3 [^{3}H]Paroxetine binding in *h*SERT OVR and Wt mice: amygdala, thalamic and hypothalamic regions

	Male							Female						
	Wild-type			hSERT OVR			%	Wild-type			hSERT OVR			%
	Mean		s.e.m	Mean		s.e.m	difference	Mean		s.e.m	Mean		s.e.m	difference
Amygdala														
Medial	86	±	12	82	±	7	**-5**	91	±	13	103	±	9	**13**
Basolateral	87	±	12	83	±	7	**-4**	99	±	11	92	±	7	**-7**
Central	83	±	11	80	±	7	**-4**	80	±	10	96	±	6	**20**
Thalamic Nuclei														
Anterior	56	±	5	61$^{\#}$	±	6	**11**	59	±	8	76$^{\#}$	±	7	**28**
Mediodorsal	43	±	7	51$^{\#}$	±	4	**21**	56**	±	7	84**$^{\#}$	±	9	**51**
Venterolateral	15	±	5	22	±	5	**5**	50***	±	9	45***	±	5	**-10**
Hypothalamic Nuclei														
Anterior	81	±	8	88	±	5	**8**	93*	±	8	114*	±	11	**21**
Venterolateral	69	±	7	84	±	5	**21**	84	±	9	101	±	16	**19**

*Effect of hSERT over-expression on [^{3}H]Paroxetine binding in amygdala, thalamic and hypothalamic regions. Data are expressed as mean ± s.e.m and % difference between hSERT OVR and Wt animals. Data were analysed using 2-way ANOVA. *denotes p<0.05, **denotes p<0.01, **denotes p<0.01 significant gender difference. $^{\#}$denotes p<0.05 significant genotype effect.*

Table 3.2.4 [^{3}H]Paroxetine binding in *h*SERT over-expressing and Wild-type mice: hippocampal regions

	Male							Female						
	Wild-type			hSERT OVR			%	Wild-type			hSERT OVR			%
	Mean		s.e.m	Mean		s.e.m	difference	Mean		s.e.m	Mean		s.e.m	difference
Molecular Layer	103	±	6	101	±	7	**-2**	103	±	13	105	±	7	**2**
Dorsal Subiculum	63	±	4	76$^{\#\#}$	±	9	**20**	56	±	9	78$^{\#\#}$	±	4	**40**†
Dentate PO	22	±	7	36$^{\#}$	±	6	**58**	38**	±	3	48**$^{\#}$	±	6	**27**
Dorsal CA1	48	±	6	61$^{\#\#}$	±	5	**27**	61**	±	5	80**$^{\#\#}$	±	4	**31**
CA2	66	±	6	70	±	6	**5**	85**	±	8	92**	±	8	**8**
Ventral CA1	63	±	8	67	±	5	**6**	92***	±	8	104***	±	8	**13**
Ventral Subiculum	79	±	7	89	±	7	**13**	90	±	9	96	±	8	**6**
CA3	56	±	6	64	±	7	**14**	63	±	6	80	±	6	**27**

*Effect of hSERT over-expression on [^{3}H]Paroxetine binding in hippocampal regions. Data are expressed as mean ± s.e.m and % difference between hSERT OVR and Wt animals. Data were analysed using 2-way ANOVA. **denotes p<0.01, ***denotes p<0.001 significant gender difference. $^{\#}$denotes p<0.05, $^{\#\#}$denotes p<0.01 significant genotype effect. †denotes p<0.05 signficant gender x genotype interaction.*

Table 3.2.5 [^{3}H]Paroxetine binding in *h*SERT OVR and wild-type mice: raphé, mesocorticolimbic and non-specific regions

	Male							Female						
	Wild-type			hSERT OVR			%	Wild-type			hSERT OVR			%
	Mean		s.e.m	Mean		s.e.m	difference	Mean		s.e.m	Mean		s.e.m	difference
Raphé														
Dorsal	90	±	9	107$^{\#}$	±	11	**19**	74	±	7	97$^{\#}$	±	4	**32**
Median	60	±	8	84$^{\#\#}$	±	8	**40**	59	±	4	81$^{\#\#}$	±	7	**38**
Paramedian	47	±	9	58	±	8	**23**	53	±	5	56	±	4	**6**
Mesocorticolimbic System														
Ventral Tegmental Area	30	±	5	53$^{\#\#}$	±	7	**79**	53**	±	5	69**$^{\#\#}$	±	9	**30**
Nucleus Accumbens	57	±	4	70	±	3	**23**	71	±	5	73	±	7	**3**
Non-specific														
Septal Nucleus	77	±	8	84$^{\#\#}$	±	6	**8**	68	±	5	101$^{\#\#}$	±	5	**48**†
Bed Nucleus of the Stria Terminalis	55	±	5	71	±	9	**28**	82*	±	6	94*	±	11	**15**
Lateral Habenula	52	±	8	61	±	6	**18**	63	±	5	45	±	8	**-28**
Periaqueductal Grey	68	±	8	78	±	7	**13**	76	±	12	93	±	8	**22**

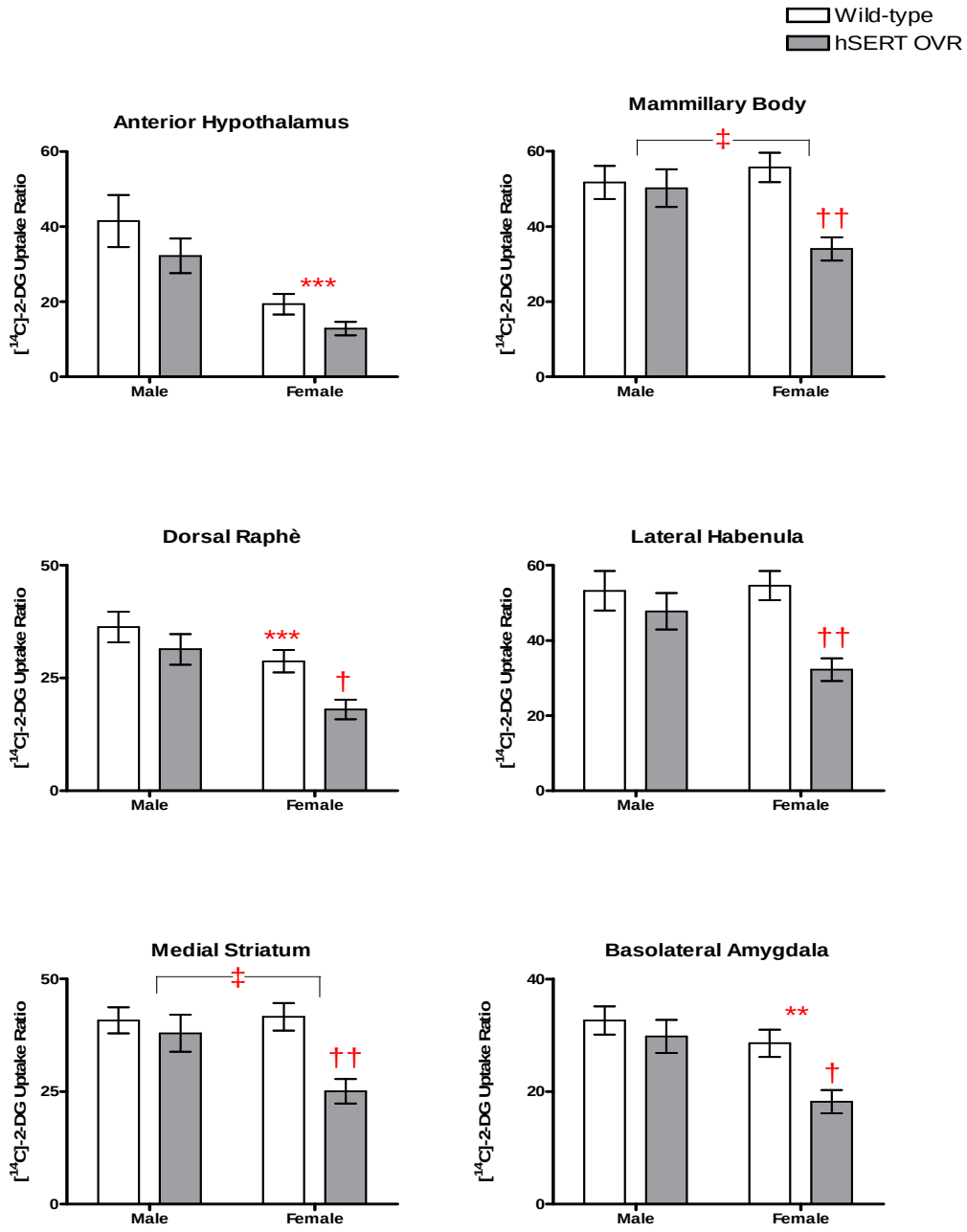
*Effect of hSERT over-expression on [^{3}H]Paroxetine binding in raphé, mesocorticolimbic and non-specific regions. Data are expressed as mean ± s.e.m and% difference between hSERT OVR and Wt animals. Data were analysed using 2-way ANOVA. **denotes p<0.01. $^{\#}$ denotes p<0.05, $^{\#\#}$ denotes p<0.01 signficant genotype effect. †denotes p<0.05 signficant gender x genotype interaction.*

2.3 Constitutive LCMRglu

In all brain regions LCMRglu tended to be lower in females than in males and this was confirmed by the significant gender effect observed in 34 of the 47 brain regions analysed. The most marked difference between the sexes was present in the anterior hypothalamus (mean 40% lower in females). LCMRglu was significantly lower in females as compared to males in all hippocampal subregions (min. effect -14%, max. effect -26%) as well as in all amygdala (min. -23%, max. -26%) and raphé (min. -21%, max. -26%) nuclei.

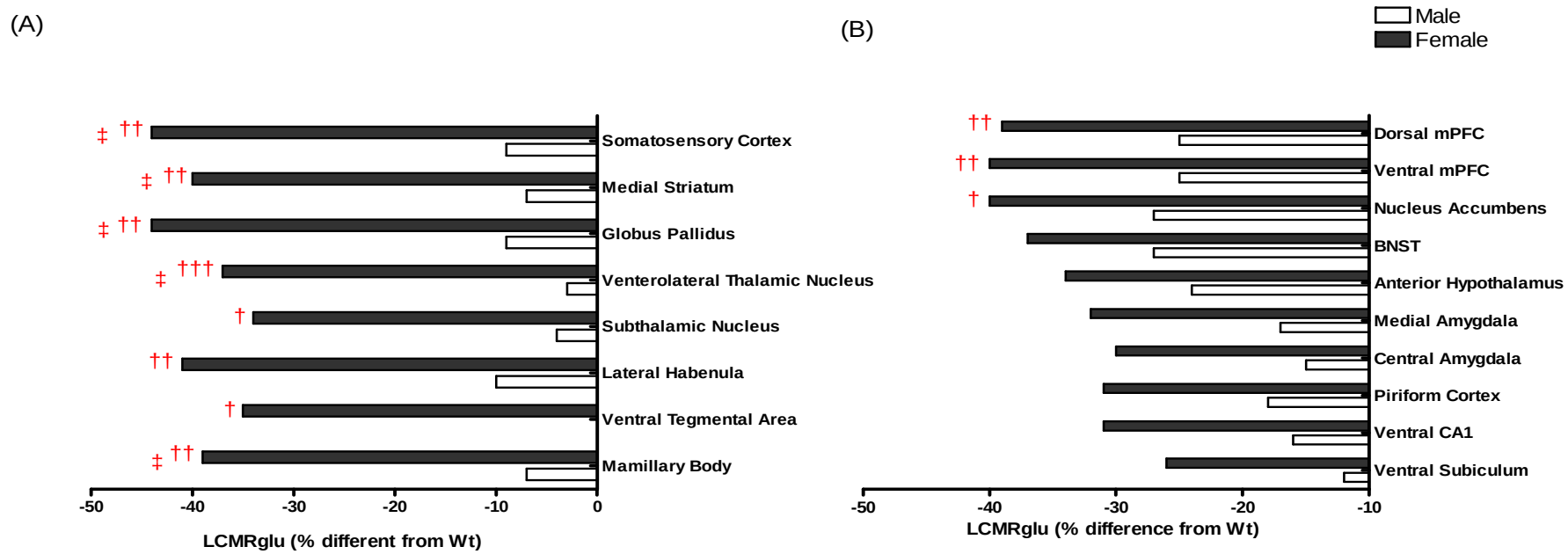
In both male and female animals *h*SERT OVR produced a tendency towards decreased LCMRglu in the majority of brain regions. However, these observed decreases only reached significance in female animals, occurring in 34 of the 47 regions of interest (ROI) analysed. In females, *h*SERT OVR resulted in significantly decreased LCMRglu in all cortical (min. -31%, max. -44%), raphé nuclei (min. -28%, max -29%), amygdala nuclei (min. -20%, max. -28%) and all hippocampal subfields (min. -16%, max. -31%) except the dentate PO. There was no evidence for a significant effect of *h*SERT OVR on LCMRglu in any ROI in males. This suggested that the ability of *h*SERT OVR to modify constitutive LCMRglu was influenced by gender. Figures 3.2.6 and 3.2.7 show representative examples of how constitutive LCMRglu is affected by gender and *h*SERT over-expression as well as how these factors interact. Despite the widespread trend for a modulatory influence of gender on the cerebral hypo-metabolism induced by *h*SERT OVR the conservative nature of 2-WAY ANOVA meant that a significant gender x *h*SERT OVR interaction was only detected in 4 brain regions; the medial striatum (26% greater reduction in females), globus pallidus (25% greater reduction in females), ventrolateral thalamus (32% greater reduction in females) and the mammillary body (27% greater reduction in females). In both male and female animals there was no correlation between the magnitude of the increased [^{3}H]paroxetine binding observed in *h*SERT OVR animals and the changes observed in constitutive LCMRglu on a regional basis. For detailed LCMRglu data in all 47 ROI analysed see Tables 3.2.6 to 3.2.11. The plasma data for mice involved in this experiment are also shown in Appendix 1 (Table A1.1)

Figure 3.2.6 Effect of gender and *h*SERT OVR on constitutive LCMRglu



*Influence of gender and hSERT OVR on constitutive LCMRglu in 6 representative brain regions. Data represent mean ± s.e.m. Data were analysed using 2-WAY ANOVA. *denotes p<0.05, **denotes p<0.01, ***denotes p<0.001 significant gender effect. †denotes p<0.05, ††denotes p<0.01 significant hSERT OVR effect (within gender with Bonferroni correction applied). ‡denotes p<0.05 significant gender x hSERT OVR interaction. In several brain regions constitutive LCMRglu is significantly decreased in females as compared to males (e.g. dorsal raphé).* h*SERT over-expression significantly decreases constitiutive LCMRglu in several limbic brain regions in females but has no effect in males (e.g. mamillary body).*

Figure 3.2.7 Influence of Gender on *h*SERT OVR mediated alterations in constitutive LCMRglu



Effect of hSERT OVR on constitutive LCMRglu in male and female mice. Data shown as % difference in the [^{14}C]-Uptake ratio from appropriate Wt control. Figures illustrate representative regions for the (A) maxiumum and (B) minimum gender difference in the effect of hSERT over-expression upon LCMRglu. †denotes p<0.05, ††denotes p<0.01 significant hSERT effect (within gender Bonferroni correction). ‡denotes p<0.05 significant gender x hSERT OVR interaction (2-WAY ANOVA). hSERT over-expression results in a significantly more pronounced constitutive hypometabolism in females as compared to males in a number of limbic brain regions (e.g. somatosensory cortex, globus pallidus and mamillary body).

Table 3.2.6 Constitutive LCMRglu in *h*SERT OVR and Wt mice: cortical regions

	Male			Female			% Gender difference		
	Wt	***h*SERT OVR**	**%**	**Wt**	***h*SERT OVR**	**%**	**Wt**	***h*SERT OVR**	***h*SERT Effect**
Cortex									
Orbitofrontal	58 ± 6	50 ± 5	**-14**	43 ± 4	31 ± 3	**-36**†	**-21****	**-29****	**-22**
Frontal	43 ± 5	36 ± 4	**-16**	44 ± 3	25 ± 2	**-44**††	**-4**	**-37**	**-28**
Anterior Cingulate	46 ± 5	38 ± 4	**-15**	43 ± 3	27 ± 3	**-38**††	**-10**	**-31**	**-23**
Dorsal medial Prefrontal	44 ± 6	35 ± 3	**-21**	37 ± 3	23 ± 2	**-39**††	**-22****	**-32****	**-18**
Ventral medial Prefrontal	40 ± 4	31 ± 3	**-21**	29 ± 2	17 ± 2	**-40**††	**-31*****	**-33*****	**-19**
Somatosensory	51 ± 5	46 ± 5	**-9**	55 ± 5	28 ± 3	**-44**††	**1**	**-43**	**-35**‡
Temperoparietal	51 ± 5	47 ± 5	**-8**	44 ± 4	28 ± 2	**-35**†	**-19****	**-28****	**-27**
Posterior Cingulate	49 ± 5	47 ± 5	**-6**	48 ± 4	32 ± 3	**-34**†	**-5**	**-27**	**-28**
Piriform	29 ± 3	24 ± 2	**-17**	20 ± 2	14 ± 2	**-31**†	**-34*****	**-21*****	**-14**
Entorhinal	34 ± 3	30 ± 3	**-14**	30 ± 2	19 ± 2	**-35**†	**-14***	**-26***	**-21**

*Constitutive LCMRglu in cortical regions of male and female hSERT OVR and wild-type mice. Data shown as mean ± s.e.m [^{14}C]-Uptake ratio and % change from appropriate control. The % difference between the genders (female v male) in each genotype, and the % difference in the effect of hSERT OVR on LCMRglu (female v male) is also shown. Data were analysed using 2-way ANOVA. *denotes p<0.05, **denotes p<0.01, ***denotes p<0.001 significant gender effect. ‡denotes p<0.05 significant gender x genotype interaction. †denotes p<0.05 significant hSERT effect within gender (2-way ANOVA with Bonferroni correction).*

Table 3.2.7 Constitutive LCMRglu in hSERT OVR and Wt mice: basal ganglia regions

	Male			Female			% gender difference		
	Wt	***h*SERT OVR**	**%**	**Wt**	**hSERT OVR**	**%**	**Wt**	***h*SERT OVR**	***h*SERT Effect**
Basal Ganglia									
Medial Striatum	41 ± 3	38 ± 4	**-7**	42 ± 3	25 ± 3	**-40**††	**-4**	**-32**	**-33**‡
Lateral Striatum	46 ± 4	40 ± 4	**-12**	42 ± 3	26 ± 3	**-37**††	**-13***	**-30***	**-25**
Globus Pallidus	30 ± 2	28 ± 3	**-9**	33 ± 3	18 ± 2	**-44**††	**1**	**-37**	**-35**‡
Subthalamic Nucleus	46 ± 5	45 ± 5	**-2**	50 ± 4	30 ± 3	**-34**†	**4**	**-32**	**-32**
Substantia Nigra pars Reticulata	29 ± 2	27 ± 2	**-7**	26 ± 2	17 ± 2	**-35**†	**-12***	**-26***	**-28**
Substantia Nigra pars Compacta	36 ± 3	33 ± 3	**-9**	34 ± 3	21 ± 2	**-39**††	**-6***	**-31***	**-30**

*Constitutive LCMRglu in basal ganglia regions of male and female hSERT OVR and wild-type mice. Data shown as mean ± s.e.m [^{14}C]-Uptake ratio and % change from appropriate control. The % difference between the genders (female v male) in each genotype, and the % difference in the effect of hSERT OVR on LCMRglu (female v male) is also shown. Data were analysed using 2-way ANOVA. *denotes p<0.05, **denotes p<0.01, ***denotes p<0.001 significant gender effect. ‡denotes p<0.05 significant gender x genotype interaction. †denotes p<0.05 significant hSERT effect within gender (2-way ANOVA with Bonferroni correction).*

Table 3.2.8 Constitutive LCMRglu in hSERT OVR and Wt mice: amygdala, thalamic and hypothalamic regions

	Male			Female			% gender difference		
	Wt	***h*SERT OVR**	**%**	**Wt**	***h*SERT OVR**	**%**	**Wt**	***h*SERT OVR**	***h*SERT Effect**
Amygdala									
Medial	24 ± 2	21 ± 2	**-14**	18 ± 2	12 ± 2	**-32**	**-30****	**-23****	**-18**
Basolateral	33 ± 2	30 ± 3	**-9**	29 ± 2	18 ± 2	**-36**†	**-18****	**-28****	**-27**
Central	22 ± 2	19 ± 2	**-12**	17 ± 2	12 ± 2	**-30**	**-29****	**-20****	**-18**
Thalamic Nuclei									
Anterior	47 ± 4	47 ± 5	**0**	47 ± 5	32 ± 3	**-32**	**-7**	**-24**	**-32**
Mediodorsal	54 ± 5	47 ± 4	**-14**	48 ± 4	29 ± 3	**-39**††	**-17****	**-32****	**-25**
Venterolateral	53 ± 5	50 ± 5	**-6**	56 ± 5	31 ± 3	**-46**†††	**1**	**-39**	**-40**‡
Hypothalamic Nuclei									
Anterior	37 ± 6	34 ± 4	**-9**	19 ± 3	13 ± 2	**-34**	**-55****	**-25****	**-25**
Venterolateral	28 ± 2	27 ± 3	**-3**	26 ± 2	16 ± 2	**-37**†	**-17***	**-29***	**-34**

*Constitutive LCMRglu in amygdala, thalamic and hypothalamic regions of male and female hSERT OVR and wild-type mice. Data shown as mean ± s.e.m [^{14}C]-Uptake ratio and % change from appropriate control. The % difference between the genders (female v male) in each genotype, and the % difference in the effect of hSERT OVR on LCMRglu (female v male) is also shown. Data were analysed using 2-way ANOVA. *denotes p<0.05, **denotes p<0.01, ***denotes p<0.001 significant gender effect.* ‡*denotes p<0.05 significant gender x genotype interaction.* †*denotes p<0.05 significant hSERT effect within gender (2-way ANOVA with Bonferroni correction).*

Table 3.2.9 Constitutive LCMRglu in *h*SERT OVR and Wt mice: hippocampal regions

	Male			Female			% gender difference		
	Wt	***h*SERT OVR**	**%**	**Wt**	***h*SERT OVR**	**%**	**Wt**	***h*SERT OVR**	***h*SERT Effect**
Hippocampus									
Molecular Layer	41 ± 4	34 ± 3	**-16**	32 ± 3	21 ± 2	**-35**†	**-21****	**-27****	**-19**
Dorsal Subiculum	38 ± 3	34 ± 3	**-11**	38 ± 3	25 ± 3	**-34**††	**-3**	**-26**	**-23**
Dentate PO	25 ± 1	22 ± 2	**-11**	20 ± 2	15 ± 2	**-25**	**-18****	**-15****	**-14**
Dorsal CA1	35 ± 3	30 ± 2	**-16**	28 ± 3	17 ± 2	**-38**†	**-21****	**-31****	**-22**
CA2	33 ± 3	27 ± 3	**-18**	26 ± 3	16 ± 2	**-38**†	**-22****	**-31****	**-20**
Ventral CA1	33 ± 2	28 ± 3	**-16**	24 ± 2	17 ± 2	**-31**	**-26*****	**-22*****	**-15**
Ventral Subiculum	29 ± 2	26 ± 3	**-11**	23 ± 2	17 ± 2	**-26**	**-21****	**-16****	**-15**
CA3	27 ± 2	24 ± 2	**-14**	21 ± 2	14 ± 2	**-34**	**-25*****	**-25*****	**-20**

*Constitutive LCMRglu in hippocampal regions of male and female hSERT OVR and wild-type mice. Data shown as mean ± s.e.m [^{14}C]-Uptake ratio and % change from appropriate control. The % difference between the genders (female v male) in each genotype, and the % difference in the effect of hSERT OVR on LCMRglu (female v male) is also shown. Data were analysed using 2-way ANOVA. *denotes p<0.05, **denotes p<0.01, ***denotes p<0.001 significant gender effect. ‡denotes p<0.05 significant gender x genotype interaction. †denotes p<0.05 significant hSERT effect within gender (2-way ANOVA with Bonferroni correction).*

Table 3.2.10 Constitutive LCMRglu in hSERT OVR and Wt mice: raphé and mesocorticolimbic regions

	Male			Female			% gender difference		
		*h*SERT			*h*SERT			*h*SERT	*h*SERT
	Wt	OVR	%	Wt	OVR	%	Wt	OVR	Effect
Raphé									
Dorsal	36 ± 3	31 ± 3	**-14**	29 ± 2	18 ± 2	**-37**†	**-23*****	**-29*****	**-23**
Median	46 ± 5	39 ± 4	**-15**	39 ± 3	25 ± 3	**-36**†	**-19****	**-28****	**-21**
Paramedian	45 ± 4	39 ± 4	**-13**	40 ± 3	25 ± 3	**-37**†	**-14****	**-29****	**-24**
Mesocorticolimbic System									
Ventral Tegmental Area	36 ± 3	37 ± 5	**2**	43 ± 4	28 ± 3	**-35**†	**17**	**-27**	**-37**‡
Nucleus Accumbens	43 ± 6	34 ± 3	**-21**	31 ± 2	19 ± 2	**-40**†	**-30****	**-32****	**-19**

*Constitutive LCMRglu in raphé and mesocorticolimbic regions of male and female hSERT OVR and wild-type mice. Data shown as mean ± s.e.m [^{14}C]-Uptake ratio and % change from appropriate control. The % difference between the genders (female v male) in each genotype, and the % difference in the effect of hSERT OVR on LCMRglu (female v male) is also shown. Data were analysed using 2-way ANOVA. *denotes p<0.05, **denotes p<0.01, ***denotes p<0.001 significant gender effect. ‡denotes p<0.05 significant gender x genotype interaction. †denotes p<0.05 significant hSERT effect within gender (2-way ANOVA with Bonferroni correction).*

Table 3.2.11 Constitutive LCMRglu in *h*SERT OVR and Wt mice: non-specific regions

	Male			Female			% gender difference		
	Wt	*h*SERT OVR	%	Wt	*h*SERT OVR	%	Wt	*h*SERT OVR	*h*SERT Effect
Septal Nucleus	34 ± 3	29 ± 3	**-13**	29 ± 2	18 ± 2	**-37**††	**-18****	**-29****	**-24**
BNST	27 ± 3	21 ± 2	**-21**	17 ± 2	11 ± 2	**-35**	**-41***	**-28***	**-14**
Corpus Callosum	16 ± 1	13 ± 1	**-16**	11 ± 2	8 ± 1	**-31**	**-31***	**-14***	**-15**
Lateral Habenula	53 ± 5	48 ± 5	**-10**	55 ± 4	32 ± 3	**-41**††	**2**	**-34**	**-31**‡
Mamillary Body	52 ± 4	50 ± 5	**-3**	56 ± 3	34 ± 3	**-39**††	**4**	**-31**	**-36**‡
Periaqueductal Grey	33 ± 3	26 ± 2	**-20**	23 ± 3	15 ± 2	**-34**	**-19****	**-25****	**-14**
Inferior Colliculus	73 ± 7	71 ± 10	**-3**	81 ± 7	48 ± 5	**-40**†	**14**	**-33**	**-37**
Ventral Tegmental Nucleus	46 ± 4	41 ± 4	**-12**	43 ± 3	26 ± 2	**-40**††	**-12***	**-32***	**-28**
Locus Coeruleus	41 ± 4	36 ± 4	**-13**	38 ± 3	22 ± 2	**-42**†	**-16***	**-33***	**-29**
Nucleus Tractus Solitaris	56 ± 9	44 ± 7	**-20**	37 ± 4	17 ± 1	**-52**	**-30***	**-31***	**-32**

*Constitutive LCMRglu non-specific brain regions of male and female hSERT OVR and wild-type mice. Data shown as mean ± s.e.m [^{14}C]-Uptake ratio and % change from appropriate control. The % difference between the genders (female v male) in each genotype, and the % difference in the effect of hSERT OVR on LCMRglu (female v male) is also shown. Data were analysed using 2-way ANOVA. *denotes p<0.05, **denotes p<0.01, ***denotes p<0.001 significant gender effect. ‡denotes p<0.05 significant gender x genotype interaction. †denotes p<0.05 significant hSERT effect within gender (2-way ANOVA with Bonferroni correction).*

2.4 Discussion and Conclusions

In this study we found that SERT protein expression was significantly higher in a number of functionally diverse brain regions in female as compared to male mice. There is a surprising paucity of published data on possible gender differences in SERT expression given the known role of gender in affective disorders and the multitude of reported gender differences in serotonergic system function (for review see Rubinow et al., 1998). However, evidence that the density of the high affinity [^{3}H]imipramine binding site is greater in the brains of females rats as compared to males (Ieni et al., 1985) and that β-CIT, which labels both SERT and DAT, is higher in human females than males (Staley et al., 2001) provides some support to our findings. The sex steroids may be suggested to have a possible role in determining the observed gender differences in SERT protein expression. Indeed, oestrogen has been found to stimulate both SERT gene transcription and protein expression levels (McQueen et al., 1997). However, the regional localisation of the gender differences found in our study do not correlate with those in which oestrogen increases SERT protein expression. Furthermore, testosterone has also been found to stimulate SERT expression in males (McQueen et al., 1999). Therefore, it seems unlikely that gender differences in SERT expression are directly related to the acute regulatory effects of sex steroids. Gender differences in SERT protein expression and function are likely to contribute to the reported gender differences in affective functioning, anti-depressant efficacy and in governing some of the other sexual dimorphisms reported in 5-HT system functioning. In particular, the enhanced expression of SERT in females may be central to the observation of the decreased synaptic 5-HT levels reported in females as compared to males (Gundlah et al., 1998; Jones and Lucki, 2005; Mitsushima et al., 2006).

Our data suggest that *h*SERT OVR mice have an increased level of SERT protein expression in a number forebrain regions as well as in the midbrain raphé, as detected by autoradiography using the selective SERT radioligand [^{3}H]paroxetine. Although the number of SERT binding sites was increased in *h*SERT OVR mice, the pattern of expression showed a close correlation to that of Wt animals and also corresponded with other studies using a variety of selective SERT radioligands (DeSouza and Kuyatt, 1987; Reader et al., 1998; Sharkey et al., 1991). Despite the observation that the expression of both the murine and human SERT proteins in *h*SERT OVR mice results in the presence of more than one binding site for SERT ligands (Loder, 2000), presumably due to the differing affinities of murine as compared to the human transporter for these ligands (Plenge and Mellerup, 1991), the use of

a saturating concentration of [^{3}H]paroxetine in our studies for both species of SERT proteins allows us to conclude that increased SERT protein expression levels (Bmax) rather than altered affinity is responsible for our observation of increased [^{3}H]paroxetine binding in *h*SERT OVR mice. Furthermore, as rodent SERT displays higher affinity for SERT ligands in comparison to the human transporter increased [^{3}H]paroxetine binding is unlikely to occur in *h*SERT OVR mice as a result of the presence of human SERT if expression levels are unaltered. Interestingly, the reported enhanced transcription of SERT mRNA observed in the raphé nuclei of *h*SERT OVR mice (Jennings et al., 2006) does not result in a universal increase in SERT protein expression throughout the brain. Rather, SERT expression is enhanced in a regionally heterogenous manner with the greatest increase found in anterior cortical regions, moderate increases in components of the basal ganglia, hippocampal regions and raphé, while in other regions, such as the amygdala nuclei, no increase is observed. Furthermore, there was also no correlation between the level of SERT expression observed in Wt animals and the magnitude of the increased SERT expression observed in *h*SERT OVR mice on a region-dependent basis. This suggests that the increased SERT protein expression in *h*SERT OVR mice is not directly related to the enhancement of SERT gene transcription. Rather, complicated regulatory mechanisms must exist that govern SERT protein expression levels as a function of enhance gene transcription and these must operate in a brain-region specific manner. As yet the nature of these mechanisms is not understood. However, as they directly regulate the outcome of enhanced SERT gene transcription they are likely to be central in mediating the effect of enhanced SERT transcription in *h*SERT OVR mice and possibly of the 5-HTTLPR in humans on affective, brain and serotonin system functioning.

Another interesting observation is that the approximate 2-fold increase in SERT binding in regions of the CNS of *h*SERT OVR mice shows a striking parallel to the magnitude (also approximately 2-fold) to the increase in SERT binding, mRNA expression and 5-HT re-uptake found in the brain or platelets of human individuals with the l/l genotype as compared to those of the s/l or s/s genotype (Greenberg et al., 1999; Heinz et al., 2000; Little et al., 1998). This adds further credence to the suggestion that *h*SERT OVR mice provide an accurate animal model of the human 5-HTTLPR polymorphism. However, it is also important to note that a number of other studies have reported the lack of an effect of the 5-HTTLPR on SERT binding in the brain (Parsey et al., 2006; Shioe et al., 2003; Van Dyck et al., 2004; Willeit et al., 2001). While it is interesting to speculate, with reference to our results, that the regional localisation of the binding measurements made in some of these

studies may be responsible for their negative findings it is also likely that the use of small sample sizes in some of these studies also contributes to their negative results.

In this study we found that LCMRglu was lower in all regions analysed in females as compared to males. This result contrasts to those reported by others using the quantitative method in rats which have found no gender difference in LCMRglu in the majority of brain regions (Nehlig et al., 1985) or a significantly increased rate of LCMRglu in females as compared to males (Brown et al., 1996). The reason for the disparities in these results is largely unknown. However, as Brown et al. (1996) measured LCMRglu in ovariectomised female rats with oestrogen replacement and acute administration of estradiol is known to increase LCMRglu (Namba and Sokoloff, 1984) the possible contribution of the enhanced plasma oestrogen levels present in these females can not be ruled out. Furthermore, prolonged acute stress enhances LCMRglu to a greater extent in females than in males. Therefore, use of restraint during the quantitative determination of LCMRglu in rats may also contribute in the apparent sexual dimorphisms of increased LCMRglu in females as compared to males. In contrast, the SQ 2-deoxyglucose method used in this study does not expose animals to a prolonged period of restraint stress and so the possible influence of the enhanced LCMRglu response to stress in females is negated. In addition, in our own studies using the quantitative method in Dark Agouti rats we have also found a generalised significant decrease in LCMRglu in females as compared to males in the same brain regions analysed within this study (unpublished observations), further supporting our findings in mice. As our results in Dark-Agouti rats are divergent from those reported by others in Sprague-Dawley rats (Nehlig et al., 1985) this suggests that strain and gender may interact to influence constitutive LCMRglu, although this is yet to be adequately investigated.

We also found that a life-long increase in SERT function decreases constitutive cerebral metabolism in a number of brain regions and that this effect was only significant in females. Interestingly, many of the regions in which a decreased rate of LCMRglu was found in *h*SERT OVR females as compared to Wt animals are strongly implicated in the regulation of anxiety. Pharmacological challenges associated with an anxiolytic effect, including acute diazepam and phenobarbital treatment, commonly result in decreased LCMRglu in many of the regions in which constitutive LCMRglu is decreased in *h*SERT OVR mice. These regions include the mamillary body, ventrolateral thalamus, septal nucleus, medial striatum and globus pallidus (Ableitner and Herz, 1987; Ableitner et al., 1985; Kelly et al., 1986). In addition, a number of anxiogenic pharmacological challenges have been reported to elicit

increased LCMRglu in these structures (Pratt, 1988; Ableitnet 1987) and chronic prenatal treatment with diazepam which produces an anxiolytic phenotype in the "drug-free" adult is also associated with reduced LCMRglu in many of the structures (Schroeder, 1997). Overall, our data suggest that constitutive cerebral function is decreased in several brain structures in which neuronal activity is positively correlated with anxiety. In addition, elevated LCMRglu in the lateral habenula appears to be the most robust correlate of depressive behaviour of rats and this hyper-metabolism is reversed, in parallel with the alleviation of depressive-like behaviour, by antidepressant treatment (Caldecott-Hazard et al., 1988). Therefore, it is notable that LCMRglu is also significantly decreased in the lateral habenula of *h*SERT OVR mice suggesting that hypo-metabolism in this region may contribute to their anti-depressive phenotype. Interestingly, the observation of decreased constitutive metabolism in limbic structures of female *h*SERT OVR mice parallels the reported basal fronto-limbic hypo-metabolism reported in l/l as compared to s/s allele human individuals (Graff-Guerrero et al., 2005). However, while significant hypo-metabolism was limited to frontal cortical structures in l/l human individuals we found evidence for a more widespread effect in *h*SERT OVR female mice, which also included many sub-cortical regions and the raphé nuclei. One reason for this disparity may be the lack of sample segregation on the basis of gender in the human study, which could lead to the masking of a more widespread hypo-metabolism in l/l allele females by the presence of a smaller effect in males. Indeed, it is pertinent that the hypo-metabolism reported in l/l human individuals is limited to anterior cortical regions that parallel those in which the greatest effect was detected in *h*SERT OVR females.

Interestingly, several of the regions in which hypo-metabolism was observed in *h*SERT OVR mice (including the orbitofrontal cortex, thalamus and mamillary body) have been shown to display increased metabolism in response to acutely increased 5-HT neurotransmission (Bremner et al., 1997; Cudennec et al., 1988b; Mann et al., 1996). This suggests that the decreased synaptic availability of 5-HT in *h*SERT OVR mice may be directly responsible for the decreased neuronal activity observed in these regions. However, electrolytic and neurotoxic manipulations that result in a prolonged decrease 5-HT neurotransmission fail to reduce LCMRglu in these regions (Cudennec et al., 1988a). Furthermore, a number of brain regions shown to be hypo-metabolic in *h*SERT OVR mice have been shown to display a decreased rate of metabolism in response to acutely increased 5-HT neurotransmission (McBean et al., 1999; Smith et al., 2002a; Smith et al., 2002b). These results suggest that prolonged alterations in synaptic 5-HT availability, such as those in *h*SERT OVR mice and lesioned animals, do not mirror the effects of acutely altered 5-HT levels. This most likely

reflects the occurrence of compensatory mechanisms in 5-HT neurotransmission when 5-HT availability is chronically altered. Alterations in serotonin receptor expression and function are likely to constitute some of these alterations. Therefore, it will be important to characterise the possible alterations in serotonin receptor function that may contribute to the constitutive hypo-metabolism observed in *h*SERT OVR mice.

Evidence from both [^{3}H]paroxetine binding and constitutive LCMRglu data suggest that gender modulates the influence of *h*SERT OVR on these parameters, with *h*SERT OVR having a greater effect in females than in males. The greater enhancement of [^{3}H]paroxetine binding in the CNS of females as compared to males by *h*SERT OVR suggests that direct gender modulation of the increase in SERT protein expression elicited by the increased transcription of the SERT gene may be central to the reported gender differences in the influences of the 5-HTTLPR on affective functioning in humans. However, in *h*SERT OVR mice the modulatory influence of gender on increased SERT expression was limited in its regional distribution, being significant in the dorsal subiculum and septal nucleus. In contrast, the influence of gender on the cerebral hypo-metabolism present in *h*SERT OVR animals was more widespread and did not include either the dorsal subiculum or septum. Furthermore, the regions in which LCMRglu was altered by *h*SERT OVR to a greater extent in females than in males do not receive dense innervation from either the dorsal subiculum or septum. This may suggest that the influence of gender on the hypo-metabolism in *h*SERT OVR mice may not be directly related to the influence of gender on SERT expression levels in these animals. However, it is commonly reported that regional alterations in LCMRglu in response to pharmacological challenge do not correlate with the regional distribution of receptors. This is thought to be due to both the sensitivity of the method, which localises metabolic alterations at the synapse rather than cell body, the convergence and contribution of multiple neuronal pathways to the observed alterations in LCMRglu and the behavioural effects of drug treatment which may also alter LCMRglu (e.g. by increasing locomotor activity). Therefore, it is not surprising that the gender effects of *h*SERT OVR on SERT protein expression do not correlate with those of LCMRglu. However, one other possibility is that the gender dependent modulation of secondary responses to the increased SERT expression in *h*SERT OVR animals, rather than of SERT expression itself, may be primarily responsible for the gender differences observed in the influence of *h*SERT OVR on LCMRglu. For example, gender modulation of the possible alterations in 5-HT receptor functioning that may occur in response the altered SERT function in *h*SERT OVR animals and may contribute to the gender differences noted in LCMRglu. A contention supported by

the increased alteration in 5-HT_{1A} receptor function present in SERT KO female animals as compared to males (Li et al., 2000). Overall, these data suggest that increased SERT transcription modifies constitutive cerebral function to a greater in females than in males. While this finding parallels reported gender-differences observed in humans in the effect of the 5-HTTLPR on affective functioning the possible gender difference in anxiety-like behaviour in *h*SERT OVR animals has not been investigated. However, our data suggest that *h*SERT OVR may produce a greater anxiolytic effect in females than in males.

Interestingly, we found that the rate of metabolism in the ventrolateral hypothalamus was significantly reduced in *h*SERT OVR mice. Evidence suggests that neuronal activity in this region regulates feeding behaviour as the excitation of neurones within this region with excitatory amino acids results in intense feeding behaviour (Stanley et al., 1993). Furthermore, the local application of GABA or $GABA_A$ agonists into this brain region results in decreased feeding (Kelly et al., 1977; 1979). This suggests, therefore, that *h*SERT OVR mice may eat less than Wt mice, which may contribute to their decreased body weight (Figure 1.7). Clearly, further research should be dedicated to investigating possible alterations in the feeding behaviour of hSERT OVR mice and the possible influence of altered hypothalamic neuronal function on the feeding behaviour of these mice.

In summary, the SERT expression and cerebral functional alterations present in *h*SERT OVR mice parallel those observed in l/l allele in comparison to s/s allele human individuals suggesting that *h*SERT OVR mice provide a good animal model of the 5-HTTLPR polymorphism. In addition, alterations in SERT expression and cerebral functioning observed in *h*SERT OVR mice are greater in females than in males which parallels the known gender-difference in the influence of the 5-HTTLPR polymorphism on affective functioning and the antidepressant response in humans. Alterations in 5-HT receptor expression and/or functioning in *h*SERT OVR animals are likely to occur in response to the decreased synaptic 5-HT availability in these animals and may play a role in the constitutive hypo-metabolism present in these animals. Possible alterations in 5-HT receptor functioning in *h*SERT OVR mice warrant further investigation.

3. Study 3- 5-HT_{1A} binding and function in *h*SERT over-expressing mice

3.1 Rationale

Alterations in 5-HT_{1A} receptor function are postulated to play a central role in both affective psychopathology and the response to antidepressant treatment (see section 4.4.1). Furthermore, evidence from both human and animal studies supports a central role for genetically determined SERT levels in the regulation of 5-HT_{1A} receptor expression and function. In humans the 's' polymorphism in the 5-HTLLPR region of the SERT gene, resulting in a life-long decrease in SERT functioning, is associated with decreased post-synaptic 5-HT_{1A} binding whereas 5-HT_{1A} autoreceptor binding in the raphé is unaltered (David et al., 2005). Although this suggests that post-synaptic 5-HT_{1A} receptor expression may be decreased as a result of a life-long decrease in SERT function the possible effect of the polymorphism on 5-HT_{1A} receptor function has not yet been explored. In mice where SERT function is knocked-out (KO), however, the most consistent observation has been decreased 5-HT_{1A} autoreceptor binding and function. Whereas, observations of reduced post-synaptic 5-HT_{1A} binding and function in SERT KO mice, despite consistency in their directionality, have been inconsistent in their localisation (Fabre et al., 2000a; La Cour et al., 2001; Li et al., 1999). Mechanistically, it is suggested that the decreased SERT function, both in 's' allele humans and SERT KO mice, leads to 5-HT_{1A} receptor desensitisation due to increased synaptic 5-HT levels. In line with this suggested mechanism and the findings of previous studies it may be hypothesised that 5-HT_{1A} binding and function may be increased in *h*SERT OVR mice as a result of the decreased synaptic 5-HT availability in these animals (Jennings et al., 2006).

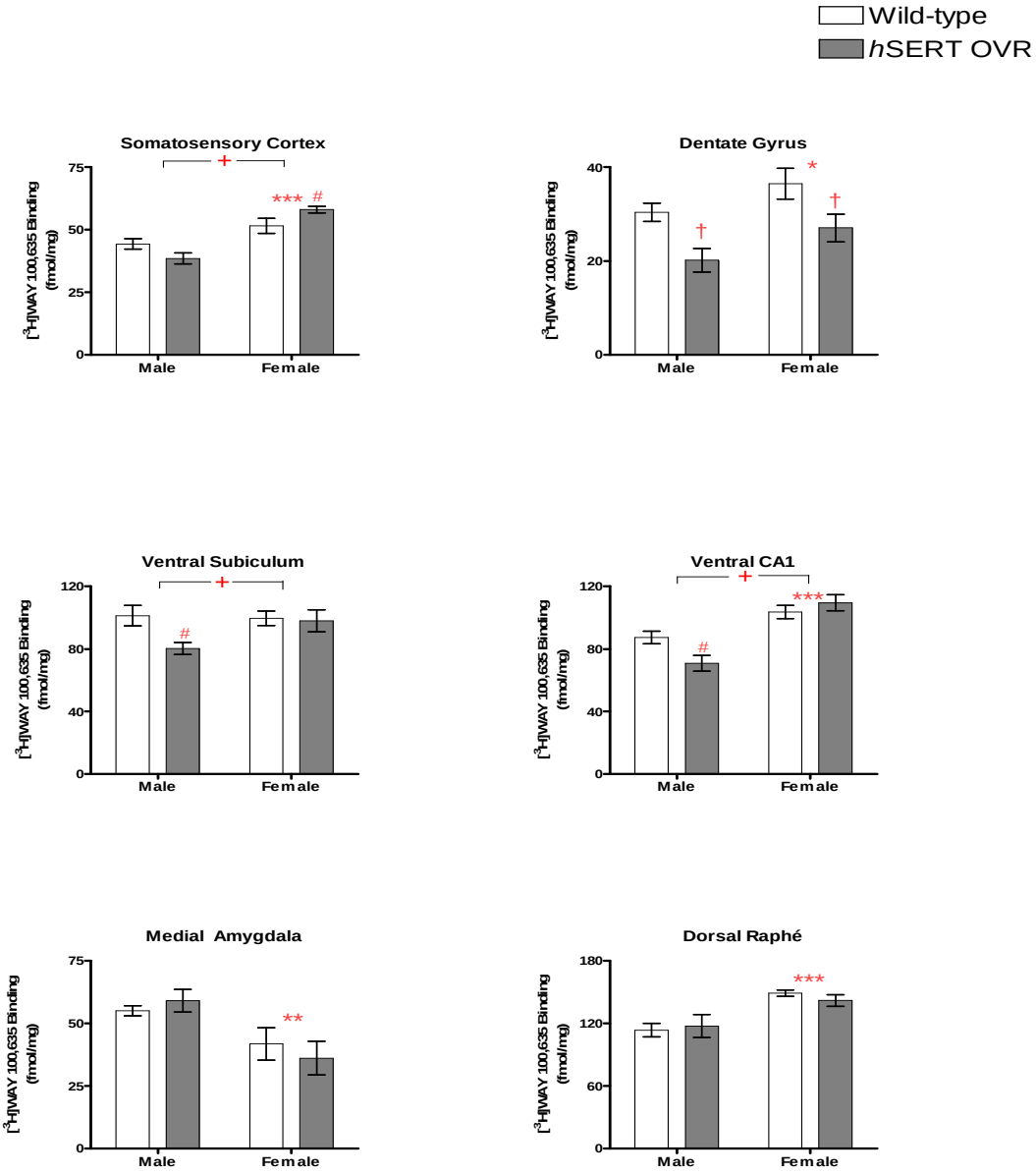
In order to further understand the alterations in serotonin receptor function that may be responsible for the reported alterations in constitutive neuronal functioning in *h*SERT OVR mice here we characterised 5-HT_{1A} binding using [^{3}H]WAY100,635 and 5-HT_{1A}-mediated functional responses, in terms of the LCMRglu response to 8-OH-DPAT, in these animals. Furthermore, as our previous studies suggest that the ability of *h*SERT OVR to modify neuronal activity is influenced by gender and other studies suggest that the ability of genetically determined SERT expression levels to modify 5-HT_{1A} receptor function may be greater in females than males (David et al., 2005; Li et al., 2000) we will investigate these parameters in animals of both genders.

3.2 5-HT_{1A} Receptor Binding

In the majority of brain regions [^{3}H]WAY100,635 binding tended to be greater in females in comparison to males. Indeed, [^{3}H]WAY100,635 binding was found to be significantly increased in females as compared to males in 23 of the 48 brain regions analysed. This gender difference was prevalent in diverse cortical regions (range +21% to +42%), multiple hippocampal subfields (range +4% to +36%) and in the dorsal (+26%) and median (+28%) raphé. The only region in which [^{3}H]WAY100,635 binding was significantly decreased in females as compared to males was in the medial amygdala (-35%). In contrast there was no evidence for a significant gender difference in 5-HT_{1A} binding in any of the hypothalamic regions analysed. Gender differences in [3H]WAY 100,635 binding for a number of representative brain regions are shown in Figures 3.3.1 and 3.3.2.

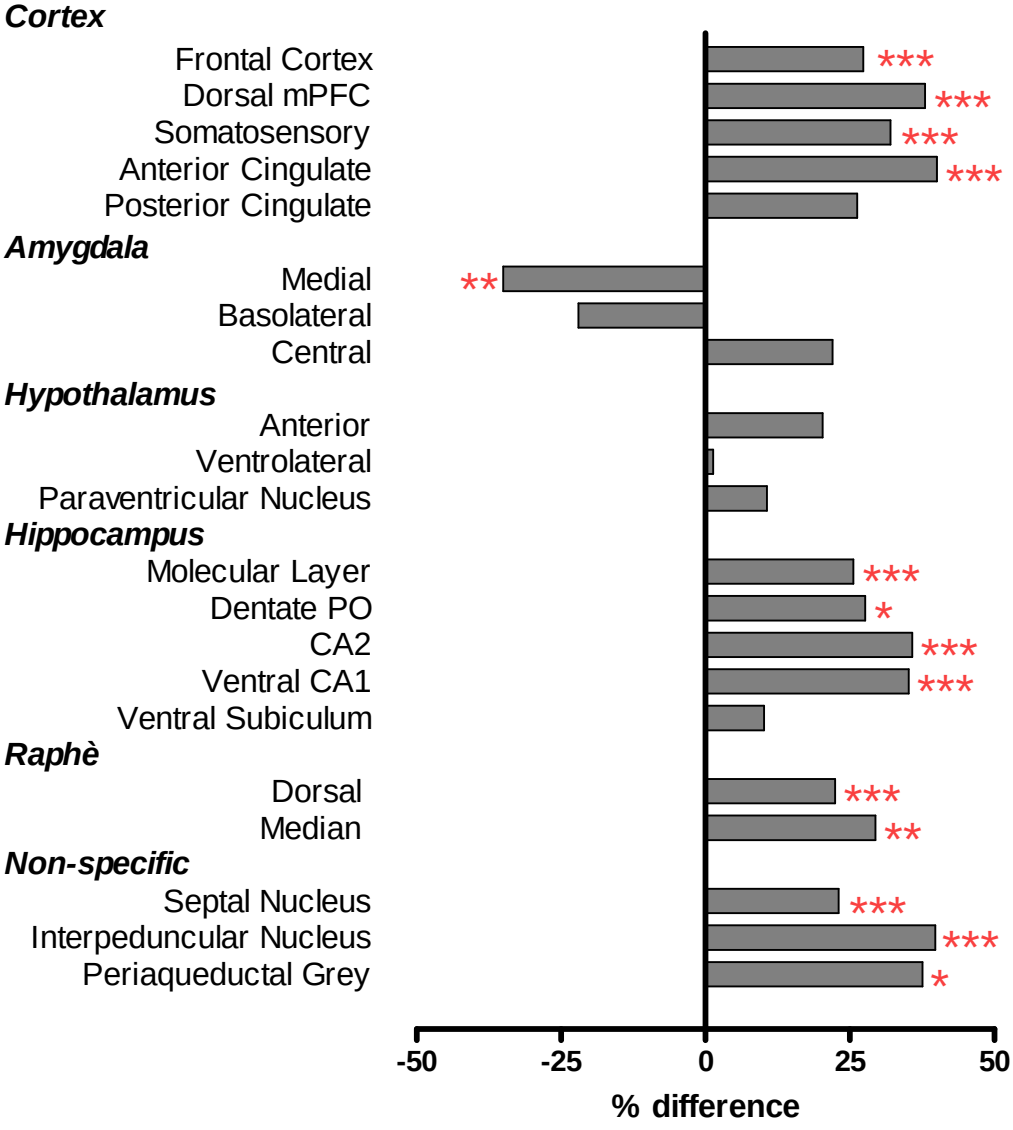
*h*SERT OVR produced a trend towards decreased [^{3}H]WAY100, 635 binding in the majority of brain regions in males, whereas this trend was more limited in females. In both males and females *h*SERT OVR produced a significant decrease in [^{3}H]WAY100,635 binding in the dentate gyrus (males: -39%, females: -17%) and dentate PO (males: -35%, females: -11%). Despite the observation that [^{3}H]WAY100,635 binding was reduced to a greater extent in males than in females in response to *h*SERT over-expression in these regions there was no significant evidence for a gender x genotype interaction. However, in two other regions, the CA2 and VCA1 subfields of the ventral hippocampus, a significant interaction was found between genotype and gender indicating that *h*SERT OVR resulted in a significant decrease in [^{3}H]WAY100,635 binding in males (CA2: -20%, VCA1 -22%) but not females (CA2: +6%, VCA1 -2%) in these regions (Figures 3.3.1 and 3.3.3). A further significant interaction between gender and genotype was identified in the somatosensory cortex where *h*SERT OVR was found to result in a significant increase in [^{3}H]WAY100,635 binding in females (+17%) and a non-significant decrease in males (-12%). Detailed data on [^{3}H]WAY100,635 binding in male and female *h*SERT OVR animals and their Wt littermates are presented in Tables 3.3.1 to 3.3.5.

Figure 3.3.1 [^{3}H]WAY100,635 binding in *h*SERT OVR and wild-type mice



*[^{3}H]WAY100,635 binding in 6 representative brain regions in male and female hSERT OVR and wild-type mice. Data shown as mean ± s.e.m. *denotes p<0.05, **denotes p<0.01 and ***denotes p<0.01 significant gender effect. †denotes p<0.05 significant genotype effect. $^{+}$denotes p<0.05 significant genotype x gender interaction (2-way ANOVA). $^{\#}$denotes p<0.05 significant genotype effect within gender (2-way ANOVA with Bonferroni Correction). [^{3}H]WAY 100,635 is significantly ihigher in several brain regions in females as compared to males (e.g dorsal raphé, medial amygdala). [^{3}H]WAY 100,635 binding is significantly decreased in the dentate gyrus of both male and female hSERT OVR mice. In addition [^{3}H]WAY100,635 binding is reduced in other hippocampal brain regions (ventral subiculum and ventral CA1) in male hSERT OVR mice but is not altered in females.*

Figure 3.3.2 Gender differences in [^{3}H]WAY100,635 binding



*Gender differences in [^{3}H]WAY100,635 binding in representative brain regions of diverse functional systems. Data shown as % difference in binding in female animals as compared to males, with data gained from both Wt and hSERT OVR animals. *denotes p<0.05, **denotes p<0.01 and ***denotes p<0.001 significant gender difference (2-way ANOVA). [^{3}H]WAY100,635 binding is significantly higher in females as compared to males in a number of brain regions.*

Figure 3.3.3 Gender modulates the influence of *h*SERT OVR on [^{3}H]WAY 100,635 binding

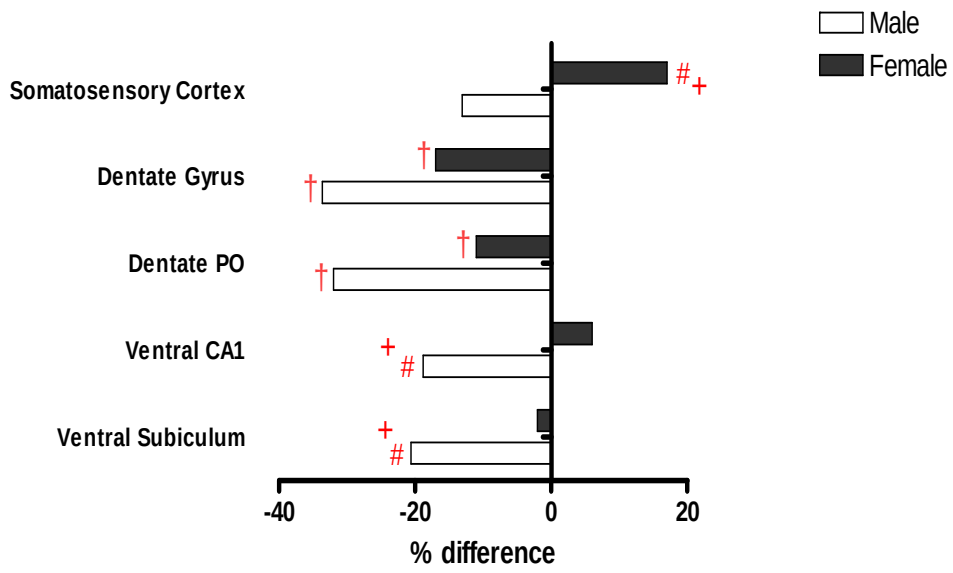


Figure showing regions in which [^{3}H]WAY 100,635 binding is significantly altered in hSERT OVR mice. Data shown as % difference in binding from appropriate Wt control. Data were analysed using 2-way ANOVA. †denotes p<0.05 significant hSERT OVR effect, $^{+}$denotes p<0.05 significant gender x genotype interaction. $^{\#}$denotes p<0.05 significant hSERT effect within gender (2-way ANOVA with Bonferroni correction). h*SERT OVR significantly increases [^{3}H]WAY100,635 binding in the somatosensory cortex, but only in female animals. In contrast [^{3}H]WAY100,635 binding is decreased in the dentate gyrus and dentate PO by* h*SERT over-expression in animals of both genders. However, in the ventral CA1 and ventral subiculum [^{3}H]WAY100,635 binding is only decreased by hSERT over-expression in male mice.*

Table 3.3.1 [^{3}H]WAY100,635 Binding in hSERT over-expressing mice: cortical regions

	Male							Female						
	Wild-type			*h*SERT OVR			%	Wild-type			*h*SERT OVR			%
	Mean		s.e.m	Mean		s.e.m	difference	Mean		s.e.m	Mean		s.e.m	difference
Cortex														
Orbitofrontal	70.4	±	5.5	65.7	±	2.5	**-7**	95.5***	±	6.2	99.2	±	3.3	**4**
Frontal	50.9	±	2.6	50.9	±	1.7	**0**	64.3***	±	3.9	68.0	±	2.6	**6**
Anterior Cingulate	34.8	±	1.5	29.0	±	1.9	**-17**	43.7***	±	4.8	46.4	±	3.5	**6**
Dorsal medial Prefrontal	39.5	±	1.7	35.5	±	1.4	**-10**	51.9***	±	4.7	54.0	±	2.2	**4**
Ventral medial Prefrontal	45.8	±	1.7	41.8	±	1.1	**-9**	59.7**	±	6.6	54.8	±	2.3	**-8**
Somatosensory	44.2	±	1.5	38.4	±	1.6	**-13**$^{+}$	51.5***	±	3.1	60.4	±	2.6	**17**$^{+\#}$
Frontal Piriform	78.9	±	2.2	80.8	±	3.0	**2**	92.4***	±	3.6	101.4	±	4.2	**-4**
Temperoparietal	30.7	±	1.3	25.5	±	1.8	**-17**	38.6**	±	4.6	4.6	±	3.1	**8**
Posterior Cingulate	23.8	±	1.8	19.4	±	2.9	**-18**	25.9	±	2.9	28.0	±	3.8	**8**
Piriform	46.2	±	0.4	36.0	±	1.6	**-22**	49.0**	±	3.7	51.6	±	3.7	**5**
Entorhinal	89.0	±	9.1	55.0	±	3.1	**-38**	98.6*	±	9.4	76.1	±	17.4	**-7**

*Effect of hSERT over-expression on [^{3}H]WAY100,635 binding in cortical and basal ganglia regions. Data are expressed as mean ± s.e.m and were analysed using 2-way ANOVA. *denotes p<0.05, **denotes p<0.01, ***denotes p<0.001 significant gender difference. + p<0.05 denotes significant gender x genotype interaction. $^{\#}$denotes significant genotype effect within-sex (2-way ANOVA with Bonferroni correction). n/s indicates no significant [^{3}H]WAY100,635 binding in region (t-test TB against NSB).*

Table 3.3.2 [^{3}H]WAY100,635 Binding in hSERT over-expressing mice: basal ganglia

	Male							Female						
	Wild-type			*h*SERT OVR			%	Wild-type			*h*SERT OVR			%
	Mean		s.e.m	Mean		s.e.m	difference	Mean		s.e.m	Mean		s.e.m	difference
Basal Ganglia														
Medial Striatum	6.6	±	0.7	3.4	±	1.2	**n/s**	3.6	±	3.0	2.8	±	3.9	**n/s**
Lateral Striatum	5.3	±	0.7	2.3	±	1.1	**n/s**	2.7	±	2.7	0.1	±	4.3	**n/s**
Globus Pallidus	5.9	±	0.6	2.8	±	0.9	**n/s**	3.4	±	3.1	1.5	±	4.1	**n/s**
Subthalamic Nucleus	19.9	±	1.0	17.9	±	1.0	**-10**	12.6	±	4.9	16.1	±	4.6	**27**
Substantia Nigra pars Reticulata	3.9	±	0.8	1.7	±	2.1	**n/s**	1.0	±	4.4	2.3	±	3.9	**n/s**
Substantia Nigra pars Compacta	5.2	±	0.9	6.2	±	1.6	**n/s**	5.7	±	4.7	4.7	±	4.0	**n/s**

*Effect of hSERT over-expression on [^{3}H]WAY100,635 binding in cortical and basal ganglia regions. Data are expressed as mean ± s.e.m and were analysed using 2-way ANOVA. *denotes p<0.05, **denotes p<0.01, ***denotes p<0.001 significant gender difference. + p<0.05 denotes significant gender x genotype interaction. $^{\#}$denotes significant genotype effect within-sex (2-way ANOVA with Bonferroni correction). n/s indicates no significant [^{3}H]WAY100,635 binding in region (t-test TB against NSB).*

Table 3.3.3 [^{3}H]WAY100,635 Binding in *h*SERT over-expressing mice: amygdala, thalamic and hypothalamic regions

	Male							Female						
	Wild-type			*h*SERT OVR			%	Wild-type			*h*SERT OVR			%
	Mean		s.e.m	Mean		s.e.m	difference	Mean		s.e.m	Mean		s.e.m	difference
Amygdala														
Medial	55.0	±	1.5	59.0	±	3.3	**7**	41.7**	±	6.5	31.5	±	7.2	**-25**
Basolateral	13.4	±	1.1	12.1	±	2.4	**-10**	11.5	±	4.3	5.8	±	4.2	**-50**
Central	18.2	±	2.3	16.9	±	2.9	**-7**	25.8	±	4.8	19.5	±	3.0	**-25**
Thalamic Nuclei														
Anterior	3.9	±	0.5	0.9	±	2.1	**n/s**	1.1	±	3.1	0.3	±	3.9	**n/s**
Mediodorsal	5.3	±	0.9	2.4	±	2.0	**n/s**	0.8	±	2.7	0.1	±	3.9	**n/s**
Venterolateral	4.2	±	0.6	0.2	±	2.0	**n/s**	1.9	±	3.2	1.6	±	4.7	**n/s**
Hypothalamic Nuclei														
Anterior	20.6	±	3.2	18.6	±	3.6	**-10**	25.8	±	2.9	26.9	±	2.4	**4**
Paraventricular	30.2	±	1.8	24.4	±	2.2	**-19**	31.6	±	3.1	28.5	±	3.4	**-10**
Venterolateral	15.9	±	0.7	14.2	±	1.7	**-11**	15.9	±	3.1	14.6	±	4.4	**-8**
Ventromedial	25.1	±	0.9	27.0	±	1.7	**8**	34.7	±	4.4	25.3	±	4.5	**-27**

*Effect of hSERT over-expression on [^{3}H]WAY100,635 binding in amygdala, thalamic and hypothalamic regions. Data are expressed as mean ± s.e.m and were analysed using 2-way ANOVA. **denotes p<0.01 significant gender difference. n/s indicates no significant [^{3}H]WAY100,635 binding in region (t-test TB against NSB).*

Table 3.3.4 [^{3}H]WAY100,635 Binding in *h*SERT over-expressing mice: hippocampal regions

	Male							Female						
	Wild-type			hSERT OVR			%	Wild-type			hSERT OVR			%
	Mean		s.e.m	Mean		s.e.m	difference	Mean		s.e.m	Mean		s.e.m	difference
Dorsal Hippocampus														
CA1	152.8	±	4.2	139.5	±	5.9	**-9**	159.7	±	9.3	159.7	±	6.7	**7**
CA2	137.1	±	2.9	123.1	±	3.9	**-10**	151.4**	±	6.5	142.8	±	5.7	**9**
CA3	29.7	±	0.9	23.4	±	1.6	**-21**	30.9	±	4.9	29.6	±	4.1	**-5**
DG	30.3	±	1.4	20.1	±	1.9	**-34**†	36.5*	±	3.3	30.3	±	4.1	**-17**†
Ventral Hippocampus														
Molecular Layer	85.1	±	3.3	79.0	±	4.8	**-7**	105.0***	±	2.4	104.5	±	3.1	**-1**
Dorsal Subiculum	58.4	±	2.9	46.4	±	2.6	**-20**	65.6***	±	3.6	65.2	±	6.2	**-1**
Dentate PO	43.1	±	1.4	29.3	±	1.8	**-32**†	47.9*	±	5.5	42.5	±	3.9	**-11**†
Dorsal CA1	79.0	±	2.9	75.9	±	4.5	**-4**	100.6*	±	7.8	109.0	±	7.0	**8**
CA2	85.1	±	2.8	71.8	±	2.9	**-16**	103.2***	±	3.2	110.6	±	6.5	**7**
Ventral CA1	87.3	±	2.9	70.8	±	3.7	**-19**$^{\#+}$	103.6***	±	4.2	109.5	±	5.1	**6**$^{+}$
Ventral Subiculum	101.2	±	4.8	80.3	±	2.8	**-21**$^{\#+}$	99.5	±	4.7	98.0	±	7.1	**-2**$^{+}$
CA3	49.9	±	1.0	41.5	±	2.2	**-17**	47.1	±	7.3	47.8	±	2.8	**2**

*Effect of hSERT over-expression on [^{3}H]WAY100,635 binding in hippocampal regions. Data are expressed as mean ± s.e.m and were analysed using 2-way ANOVA. *denotes p<0.05, **denotes p<0.01, ***denotes p<0.001 significant gender difference. †denotes p<0.05 significant genotype effect. +denotes p<0.05 significant gender x genotype interaction. $^{\#}$denotes significant genotype effect within-sex (2-way ANOVA with Bonferroni correction).*

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Table 3.3.5 [^{3}H]WAY100,635 Binding in *h*SERT over-expressing mice: raphé, mesocorticolimbic and non-specific regions

	Male							Female						
	Wild-type			*h*SERT OVR			%	Wild-type			*h*SERT OVR			%
	Mean		s.e.m	Mean		s.e.m	difference	Mean		s.e.m	Mean		s.e.m	difference
Raphé														
Dorsal	113.3	±	4.9	117.3	±	8.1	**4**	149.0***	±	2.9	141.9	±	5.7	**-5**
Median	36.9	±	1.0	29.7	±	2.3	**-20**	41.1**	±	3.9	44.2	±	2.9	**7**
Paramedian	20.8	±	1.0	13.1	±	1.7	**-37**	22.3	±	3.6	15.7	±	5.3	**-30**
Mesocorticolimbic System														
Ventral Tegmental Area	8.8	±	0.6	5.1	±	1.8	**n/s**	2.7	±	3.9	5.4	±	4.3	**n/s**
Non-specific														
Dorsal Endopiriform Nucleus	84.0	±	2.6	78.5	±	3.1	**-7**	95.2**	±	5.4	96.6	±	1.7	**2**
Septal Nucleus	95.5	±	3.6	90.9	±	3.7	**-5**	116.3***	±	4.9	116.6	±	6.8	**0**
Bed Nucleus of the Stria Terminalis	30.8	±	1.2	31.7	±	31.7	**3**	26.7	±	1.7	31.5	±	4.8	**10**
Lateral Habenula	4.8	±	1.8	1.3	±	2.0	**n/s**	1.4	±	1.9	0.9	±	2.3	**n/s**
Interpeduncular Nucleus	117.5	±	3.3	104.9	±	6.7	**7**	151.6***	±	6.5	160.6	±	8.8	**6**
Periaqueductal Grey	25.1	±	1.5	16.6	±	1.1	**1**	28.5*	±	3.4	27.2	±	3.9	**-5**
Inferior Colliculus	23.0	±	0.7	17.6	±	3.8	**4**	31.7	±	2.8	23.8	±	5.2	**-25**

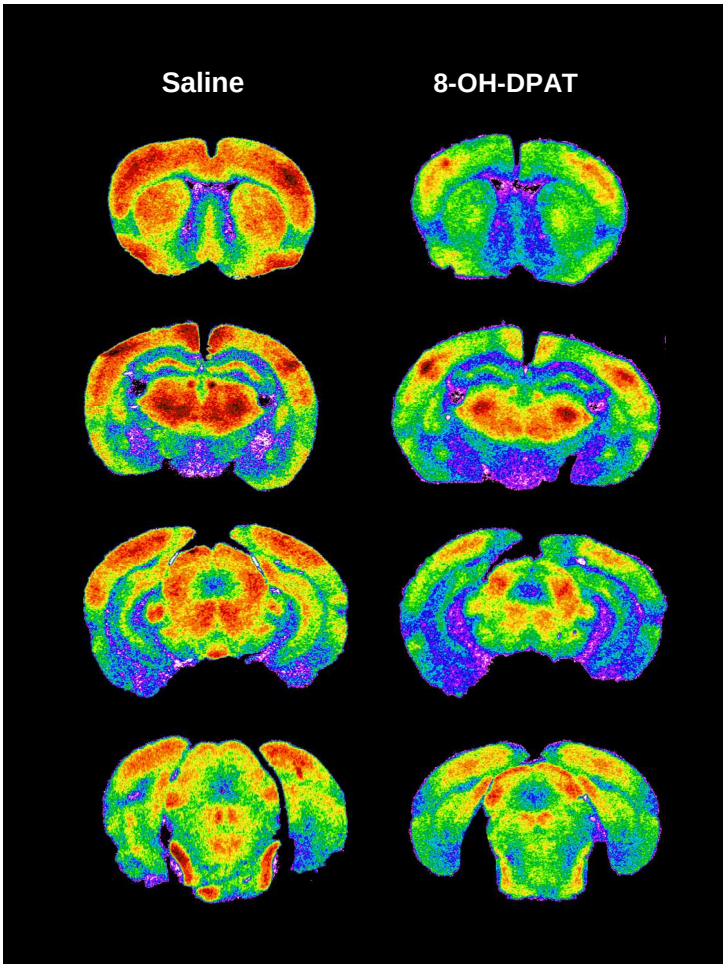
*Effect of hSERT over-expression on [^{3}H]WAY100,635 binding in raphé, mesocorticolimbic and non-specific regions. Data are expressed as mean ± s.e.m and were analysed using 2-way ANOVA. *denotes p<0.05, **denotes p<0.01, ***denotes p<0.001 significant gender difference. n/s indicates no significant [^{3}H]WAY100,635 binding in region (t-test TB against NSB).*

3.3 LCMRglu

Acute 10mg.kg^{-1} 8-OH-DPAT produced widespread decreases in LCMRglu in all experimental groups. Figure 3.3.4 shows representative autoradiograms demonstrating that 8-OH-DPAT treatment results in a wide-spread reduction in [^{14}C]-2-DG isotope accumulation. These decreases were most extensively observed in cortical regions (male: min. effect -28%, max. effect -46%; female: min. -30%, max. -51%), the hippocampus (male: min. -31%, max. -51%; female: min. -42%, max. -54%) and the raphé nuclei (male: min. -35%, max. -41, female: min. -38%, max. -52%). In Wt males these decreases reached significance in 37 of the 47 ROI analysed. In Wt females the LCMRglu decreases produced by 8-OH-DPAT in were both more pronounced and widespread than those seen in males with 44 of the 47 areas reaching significance. This suggested that the response to 8-OH-DPAT was greater in Wt females as compared to Wt males. Indeed, a significant gender x 8-OH-DPAT interaction was identified in 5 ROI where the LCMRglu response to 8-OH-DPAT was greater in females than in males (see Figures 3.3.5 and 3.3.6 for representative examples). A significantly greater response in females was noted in the globus pallidus (68 % greater), lateral habenula (100% greater), ventral tegmental area (181% greater), mamillary body (75% greater) and the inferior colliculus (53% greater). This finding is in agreement with the enhanced 5-HT$_{1A}$ binding noted in females as compared to males. In contrast to the observations made in Wt animals there was no evidence for a significant difference in the LCMRglu response to 8-OH-DPAT between male and female *h*SERT OVR mice in any ROI.

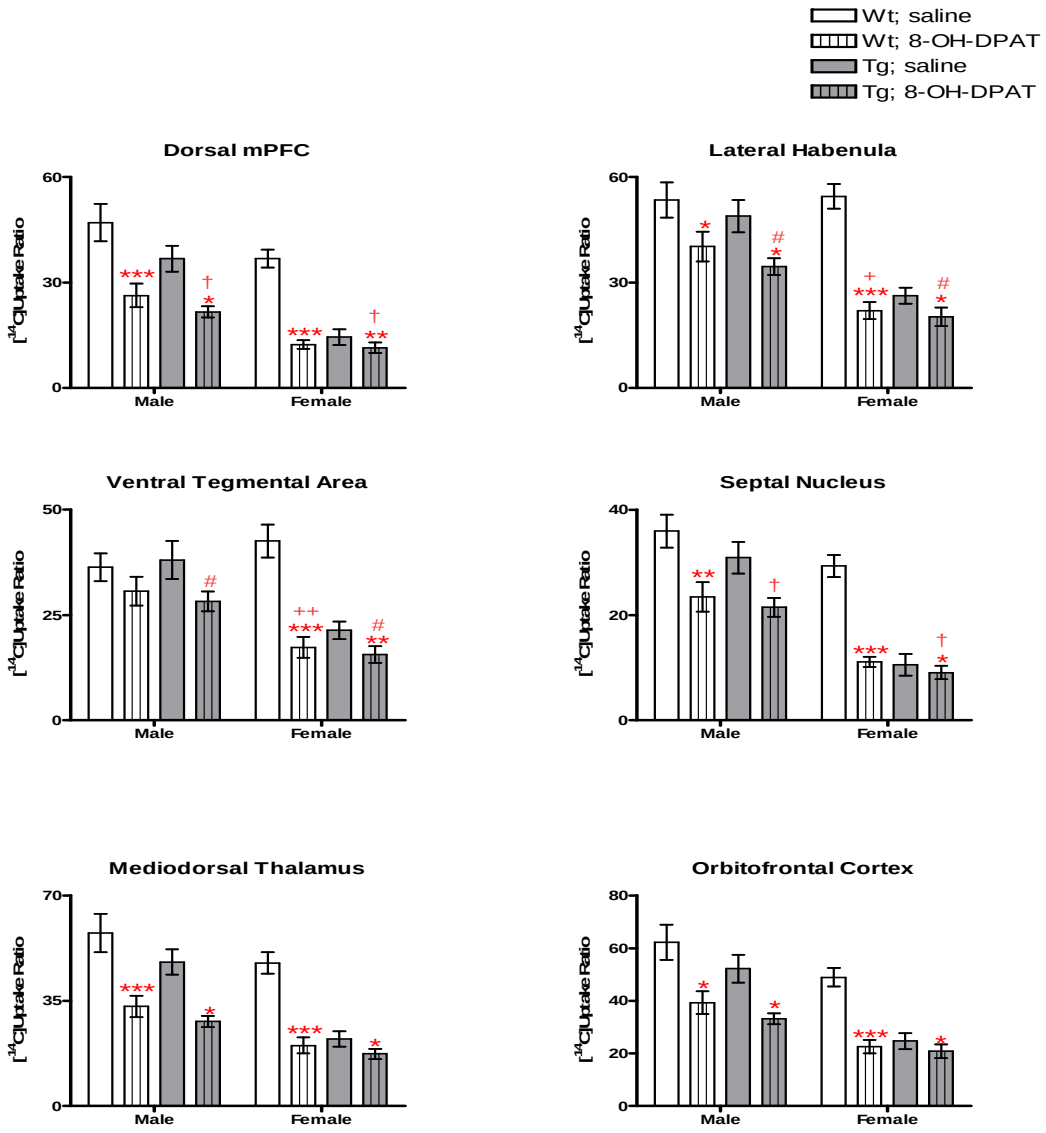
In males the LCMRglu response to 8-OH-DPAT appeared to be of a similar magnitude in the majority of brain regions in Wt and *h*SERT OVR animals. In contrast, in females the LCMRglu response to 8-OH-DPAT appeared to be reduced in the majority of brain regions in *h*SERT OVR in comparison to Wt animals. *h*SERT OVR was found to significantly reduce, to a similar extent in both males and females, the LCMRglu response to 8-OH-DPAT in 4 of the 47 ROI. These areas were the DmPFC (males -7%, females -24%), VmPFC (male -13%, female -13%), the nucleus accumbens (male -21%, female -35%) and the septal nucleus (male -14%, female -24%).

Figure 3.3.4 Autoradiograms of the LCMRglu response to 8-OH-DPAT



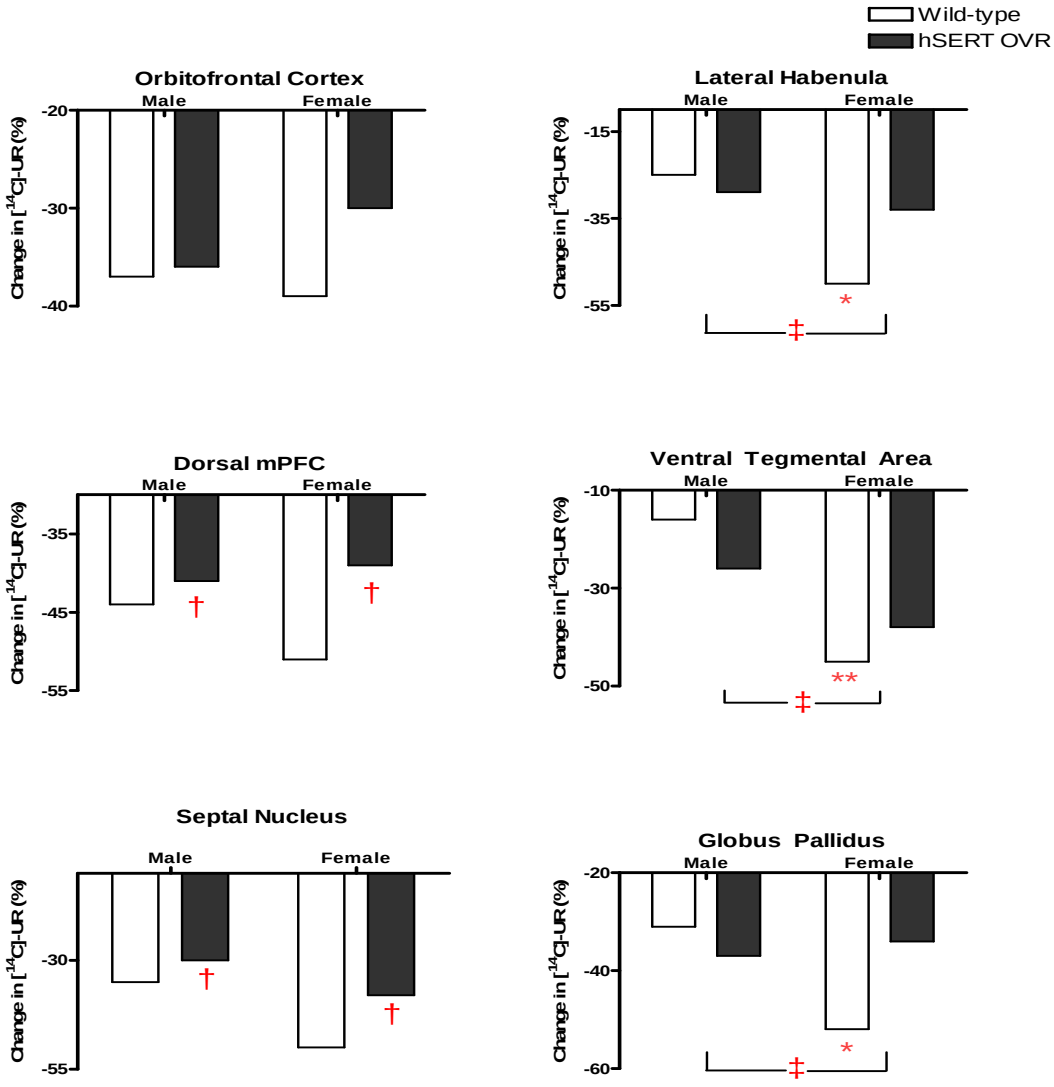
Representative "false-colour" autoradiographic images at the level of the caudate, dorsal hippocampus, ventral hippocampus and raphé in saline-treated and 8-OH-DPAT-treated wild-type male mice. High levels of tissue isotope accumulation are shown as "warm" colours (red/orange) whilst low isotope tissue accumulation is depicted by "cold" colours (green/blue). Note in particular how 8-OH-DPAT causes a wide-spread reduction in isotope accumulation.

Figure 3.3.5 LCMRglu responses to 8-OH-DPAT in Wild-type and *h*SERT OVR mice



*LCMRglu responses in 8 representative brain regions in wild-type and hSERT OVR male and female mice. Data shown as mean ± s.e.m [^{14}C]-uptake ratio. *denotes p<0.05, **denotes p<0.01 and ***denotes p<0.001 significant 8-OH-DPAT effect (within genotype 2-way ANOVA with Bonferroni correction). $^{+}$denotes p<0.05 significant gender x 8-OH-DPAT interaction (within genotype 2-way ANOVA). $^{\#}$denotes p<0.05 significant gender x genotype x 8-OH-DPAT interaction and †denotes p<0.05 signficant genotype x 8-OH-DPAT interaction in both males and females (Univariate ANOVA). 8-OH-DPAT results in significant decreases in LCMRglu in a number of brain regions, in animals of both gender and genotype. The response to 8-OH-DPAT is greater in wild-type females as compared to males in a number of brain regions (e.g. lateral habenula). The response to 8-OH-DPAT is significantly attenuated in a number of brain regions by hSERT over-expression.*

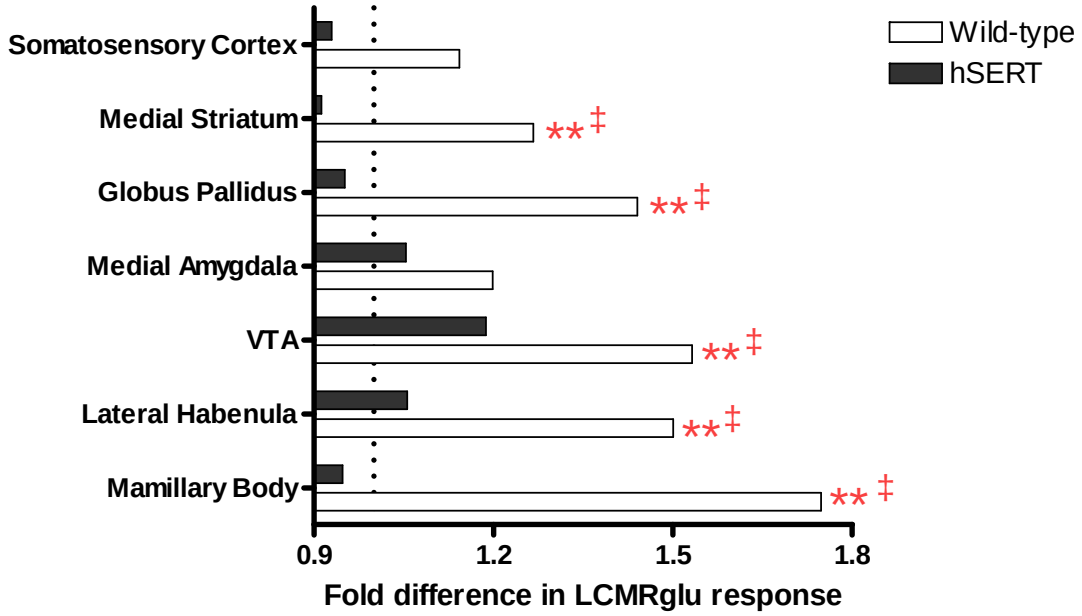
Figure 3.3.6 Effect of *h*SERT OVR on the LCMRglu response to 8-OH-DPAT



*Effect of hSERT OVR on the LCMRglu response to 10mg.kg^{-1} 8-OH-DPAT in male and female mice. Data for 6 representative brain regions shown as % change in the [^{14}C]-Uptake ratio ([^{14}C]-UR) in 8-OH-DPAT-treated animals relative to the appropriate saline control. Data analysed using Univariate ANOVA. †denotes p<0.05 significant hSERT OVR x 8-OH-DPAT interaction. ‡denotes p<0.05 significant sex x hSERT OVR x 8-OH-DPAT interaction. *denotes p<0.05, **denotes p<0.01 significant gender x 8-OH-DPAT interaction (2-WAY ANOVA within genotype). 8-OH-DPAT effect on LCMRglu was significant in all regions.The LCMRglu response to 8-OH-DPAT is significantly greater in wild-type females as compared to males in a number of brain regions (e.g. ventral tegmental area, globus pallidus). The response to 8-OH-DPAT is significantly reduced by hSERT over-expression in a number of brain regions, in both males and females (e.g. Dorsal mPFC, Septal nucleus). In some brain regions the LCMRglu response to 8-OH-DPAT is attenuated only in females (e.g. lateral habenula).*

In addition, 6 other brain regions were identified in which a significant gender x *h*SERT OVR x 8-OH-DPAT interaction was found. This interaction was observed in functionally diverse brain regions which included the lateral habenula, mammillary body, the VTA and several components of the basal ganglia (medial striatum, subthalamic nucleus, and globus pallidus). In each of these regions the LCMRglu response to 8-OH-DPAT appeared to be reduced in female animals by *h*SERT over-expression but not in males (Figure 3.3.7 for representative regions). In 4 of these regions the LCMRglu response to 8-OH-DPAT was previously found to be significantly greater in Wt female animals as compared to Wt males. However, in *h*SERT OVR animals there was no evidence for a greater effect of 8-OH-DPAT on LCMRglu in females as compared to males in any of these regions. This suggests that *h*SERT OVR reduced the LCMRglu alterations induced by 8-OH-DPAT in these regions in females to a similar level as that observed in males. Detailed data on the effects of *h*SERT over-expression on the LCMRglu response to 8-OH-DPAT are shown in Tables 3.3.6 to 3.3.11. The plasma data for animals involved in this experiment are also shown in Appendix 1, Table A1.2).

Figure 3.3.7 Attenuation of Gender differences in the LCMRglu response to 8-OH-DPAT by *h*SERT OVR



*hSERT OVR attenuates the enhanced response to 8-OH-DPAT found in wild-type females as compared to wild-type males. Data shown as fold difference in the LCMRglu ([^{14}C]-Uptake ratio) response to 8-OH-DPAT of females as compared to males. Dashed line represents 1.0 fold signifying no gender difference in the response to 8-OH-DPAT. **denotes p<0.01 significant gender x 8-OH-DPAT interaction (2-way ANOVA within genotype). ‡denotes p<0.05 significant gender x genotype x 8-OH-DPAT interaction (Univariate ANOVA).*

Table 3.3.6 LCMRglu response to 8-OH-DPAT in *h*SERT over-expressing mice: cortical regions

	Male						Female					
	Wild-type			*h*SERT OVR			Wild-type			*h*SERT OVR		
	Saline	8-OH-DPAT	%	Saline	8-OH-DPAT	%	Saline	8-OH-DPAT	%	Saline	8-OH-DPAT	%
Cortex												
Orbitofrontal	62 ± 6	39 ± 4	**-37***	52 ± 5	33 ± 2	**-36***	49 ± 4	30 ± 4	**-39*****	33 ± 3	24 ± 3	**-30***
Frontal	46 ± 5	33 ± 3	**-28***	38 ± 4	26 ± 2	**-31**	44 ± 3	25 ± 4	**-43*****	26 ± 3	19 ± 2	**-29**
Anterior Cingulate	48 ± 5	31 ± 4	**-36****	41 ± 5	24 ± 2	**-42***	43 ± 3	21 ± 3	**-51*****	28 ± 3	17 ± 2	**-43****
Dorsal medial Prefrontal	47± 5	26 ± 3	**-44*****	37 ± 4	22 ± 2	**-41*†**	37 ± 3	18 ± 3	**-51*****	24 ± 3	15 ± 2	**-39**†**
Ventral medial Prefrontal	42 ± 4	23 ± 2	**-46*****	33 ± 3	20 ± 1	**-40*†**	29 ± 2	15 ± 2	**-46*****	18 ± 2	13 ± 1	**-33†**
Somatosensory	54 ± 4	43 ± 5	**-20**	48 ± 5	37 ± 3	**-23**	55 ± 6	38 ± 7	**-30*****	29 ± 3	25 ± 3	**-17**
Temperoparietal	54± 4	34 ± 4	**-37****	48 ± 5	33 ± 3	**-31***	44 ± 4	27 ± 4	**-38*****	30 ± 2	22 ± 3	**-30***
Posterior Cingulate	51 ± 4	32 ± 3	**-37****	49 ± 5	27 ± 2	**-45****	48 ± 4	25 ± 3	**-49*****	33 ± 3	20 ± 2	**-42****
Piriform	29 ± 3	18 ± 2	**-39**	23 ± 2	15 ± 1	**-34***	20± 2	16 ± 5	**-20**	14 ± 2	10 ± 1	**-35**
Entorhinal	35 ± 3	24 ± 3	**-32***	30 ± 2	20 ± 1	**-34***	30 ± 2	18 ± 3	**-41*****	21 ± 2	15 ± 2	**-33**

LCMRglu response to 8-OH-DPAT in cortical brain regions of wild-type and *h*SERT over-expressing mice. Data shown as mean ± s.e.m and % difference in LCMRglu between 8-OH-DPAT treated and control (saline treated) animals of the same gender and genotype. *denotes, $p<0.05$, ** denotes $p<0.01$ and ***denotes $p<0.001$ significant DPAT effect (within-genotype 2-way ANOVA with Bonferroni correction). †denotes $p<0.05$ significant genotype x 8-OH-DPAT interaction (Univariate ANOVA).

Table 3.3.7 LCMRglu response to 8-OH-DPAT in *h*SERT over-expressing mice: basal ganglia regions

	Male						Female					
	Wild-type			*h*SERT OVR			Wild-type			*h*SERT OVR		
	Saline	8-OH-DPAT	%	Saline	8-OH-DPAT	%	Saline	8-OH-DPAT	%	Saline	8-OH-DPAT	%
Basal Ganglia												
Medial Striatum	43 ± 3	33 ± 3	**-24***	40 ± 5	26 ± 2	**-35**$^{\#}$	42 ± 3	25 ± 4	**-40***$^{+}$	26 ± 3	28 ± 2	**-28**$^{\#}$
Lateral Striatum	48 ± 4	29 ± 3	**-39****	43 ± 5	25 ± 2	**-41***	42 ± 3	22 ± 3	**-48****	28 ± 3	18 ± 2	**-38***
Globus Pallidus	32 ± 3	22 ± 2	**-31***	29 ± 3	18 ± 1	**-37***$^{\#}$	33 ± 3	16 ± 2	**-52***$^{+}$	29 ± 3	18 ± 1	**-34**$^{\#}$
Subthalamic Nucleus	48 ± 4	33 ± 4	**-30**	48 ± 5	29 ± 2	**-39***$^{\#}$	50 ± 3	25 ± 4	**-49****	31± 3	21 ± 2	**-36***$^{\#}$
Substantia Nigra pars Reticulata	29 ± 2	22 ± 2	**-24**	27 ± 2	19 ± 1	**-29**	26 ± 2	13 ± 2	**-50****	18 ± 2	13 ± 2	**-33**
Substantia Nigra pars Compacta	37 ± 3	27 ± 3	**-28***	34 ± 3	23 ± 2	**-32**	34 ± 3	20 ± 3	**-43****	22 ± 2	16 ± 2	**-31**

Effect of *h*SERT over-expression on the LCMRglu response to 8-OH-DPAT in basal ganglia regions. Data shown as mean ± s.e.m and % difference in LCMRglu between 8-OH-DPAT-treated and control (saline-treated) animals of the same gender and genotype. ** denotes p<0.01 and ***denotes p<0.001 significant 8-OH-DPAT effect (within-genotype 2-way ANOVA with Bonferroni correction). +denotes p<0.05 significant gender x 8-OH-DPAT interaction (within-genotype 2-way ANOVA). $^{\#}$denotes p<0.05 significant gender x 8-OH-DPAT x genotype interaction (Univariate ANOVA).

Table 3.3.8 LCMRglu response to 8-OH-DPAT in *h*SERT over-expressing mice: amygdala, thalamic and hypothalamic regions

	Male						Female					
	Wild-type			*h*SERT OVR			Wild-type			*h*SERT OVR		
	Saline	8-OH-DPAT	%	Saline	8-OH-DPAT	%	Saline	8-OH-DPAT	%	Saline	8-OH-DPAT	(%)
Amygdala												
Medial	25 ± 2	17 ± 2	**-34****	22 ± 2	14 ± 1	**-38***	18 ± 2	10 ± 1	**-45*****	13 ± 2	8 ± 1	**-40***
Basolateral	35 ± 3	26 ± 3	**-25***	31 ± 3	22 ± 2	**-29**	29 ± 2	17 ± 3	**-40*****	19 ± 2	15 ± 1	**-29**
Central	23 ± 3	17 ± 2	**-25**	20 ± 2	14 ± 1	**-32**	17 ± 2	13 ± 3	**-22**	12 ± 2	8 ± 1	**-36**
Thalamic Nuclei												
Anterior	51 ± 5	33 ± 4	**-34***	48 ± 5	29 ± 2	**-41***	47 ± 5	26 ± 3	**-46*****	34 ± 3	21 ± 2	**-40***
Mediodorsal	58 ± 6	33 ± 3	**-42*****	48 ± 4	28 ± 2	**-41***	48 ± 4	30 ± 3	**-43*****	32 ± 2	21 ± 2	**-34***
Venterolateral	56 ± 5	41 ± 5	**-27**	29 ± 3	19 ± 2	**-32**	56 ± 5	35 ± 5	**-38*****	34 ± 2	28 ± 3	**-19**
Hypothalamic Nuclei												
Anterior	43 ± 7	19 ± 2	**-55*****	32 ± 4	15 ± 1	**-54***	19 ± 3	10 ± 2	**-47***	14 ± 2	9 ± 1	**-40***
Venterolateral	31 ± 3	18 ± 2	**-40****	29 ± 3	19 ± 2	**-32**	26 ± 2	13 ± 2	**-51*****	32 ± 3	28 ± 3	**-39***

Effect of *h*SERT over-expression on the LCMRglu response to 8-OH-DPAT in amygdala, thalamic and hypothalamic regions. Data shown as mean ± s.e.m and % difference in LCMRglu between 8-OH-DPAT-treated and control (saline-treated) animals of the same gender and genotype. ** denotes $p<0.01$ and ***denotes $p<0.001$ significant 8-OH-DPAT effect (within-genotype 2-way ANOVA with Bonferroni correction).

Table 3.3.9 LCMRglu response to 8-OH-DPAT in *h*SERT over-expressing mice: hippocampal regions

	Male						Female					
	Wild-type			*h*SERT OVR			Wild-type			*h*SERT OVR		
	Saline	**8-OH-DPAT**	**%**	**Saline**	**8-OH-DPAT**	**%**	**Saline**	**8-OH-DPAT**	**%**	**Saline**	**8-OH-DPAT**	**%**
Hippocampus												
Molecular Layer	41 ± 4	26 ± 3	**-38*** **	34 ± 3	22 ± 2	**-38***	32 ± 3	17 ± 2	**-49*** **	21 ± 2	14 ± 2	**-40***
Dorsal Subiculum	38 ± 3	24 ± 3	**-39*** **	34 ± 3	20 ± 1	**-44** **	38 ± 3	18 ± 3	**-52*** **	25 ± 3	14 ± 2	**-50*** **
Dentate PO	25 ± 1	15 ± 1	**-38*** **	22 ± 2	12 ± 1	**-47** **	20 ± 2	10 ± 2	**-53*** **	15 ± 2	8 ± 1	**-52*** **
Dorsal CA1	35 ± 3	23 ± 7	**-36** **	30 ± 2	18 ± 1	**-41** **	28 ± 3	14 ± 2	**-52*** **	17 ± 2	11 ± 2	**-43***
CA2	33 ± 3	21 ± 2	**-36** **	27 ± 3	18 ± 2	**-40***	26 ± 3	13 ± 2	**-51*** **	16 ± 2	10 ± 1	**-43***
Ventral CA1	33 ± 2	20 ± 2	**-39*** **	28 ± 3	18 ± 2	**-38***	24 ± 2	12 ± 2	**-50*** **	17 ± 2	11 ± 1	**-42** **
Ventral Subiculum	29 ± 2	20 ± 2	**-31** **	26 ± 3	18 ± 1	**-35***	23 ± 2	12 ± 2	**-48*** **	17 ± 2	11 ± 1	**-43***
CA3	27 ± 2	15 ± 2	**-44*** **	24 ± 2	12 ± 1	**-51** **	21 ± 2	10 ± 2	**-54*** **	14 ± 2	8 ± 3	**-48** **

Effect of *h*SERT over-expression on the LCMRglu response to 8-OH-DPAT in hippocampal regions. Data shown as mean ± s.e.m and % difference in LCMRglu between 8-OH-DPAT-treated and control (saline-treated) animals of the same gender and genotype. ** denotes $p<0.01$ and ***denotes $p<0.001$ significant 8-OH-DPAT effect (within-genotype 2-way ANOVA with Bonferroni correction).

Table 3.3.10 LCMRglu response to 8-OH-DPAT in hSERT over-expressing mice: raphé and mesocorticolimbic regions

	Male						Female					
	Wild-type			*h*SERT OVR			Wild-type			*h*SERT OVR		
	Saline	8-OH-DPAT	%	Saline	8-OH-DPAT	%	Saline	8-OH-DPAT	%	Saline	8-OH-DPAT	%
Raphé												
Dorsal	36 ± 3	22 ± 2	**-40*****	33 ± 3	19 ± 1	**-41****	29 ± 2	13 ± 2	**-55*****	18 ± 2	13 ± 1	**-38****
Median	46 ± 5	30 ± 3	**-37****	41 ± 4	25 ± 2	**-40***	39 ± 3	19 ± 3	**-52*****	25 ± 3	17 ± 2	**-38****
Paramedian	45 ± 4	30 ± 3	**-35****	41 ± 4	25 ± 2	**-38***	40 ± 3	19 ± 3	**-52*****	25 ± 3	18 ± 2	**-38****
Mesocorticolimbic System												
Ventral Tegmental Area	36 ± 3	31 ± 3	**-16**	38 ± 4	28 ± 2	**-26**$^{\#}$	43 ± 4	23 ± 3	**-45***$^{++}$	28 ± 3	19 ± 2	**-38**$^{\#}$
Nucleus Accumbens	43 ± 6	27 ± 3	**-39****	35 ± 3	24 ± 1	**-31**†	31 ± 2	17 ± 2	**-46****	19 ± 2	15 ± 2	**-30**†

*Effect of hSERT over-expression on the LCMRglu response to 8-OH-DPAT in raphé and mesocorticolimbic. Data shown as mean ± s.e.m and % difference in LCMRglu between 8-OH-DPAT-treated and control (saline-treated) animals of the same gender and genotype. ** denotes p<0.01 and ***denotes p<0.001 significant 8-OH-DPAT effect (within-genotype 2-way ANOVA with Bonferroni correction). +denotes p<0.05 significant gender x 8-OH-DPAT interaction (within-genotype 2-way ANOVA). $^{\#}$denotes p<0.05 significant gender x 8-OH-DPAT x genotype interaction and †denotes p<0.05 significant genotype x 8-OH-DPAT effect in both males and females (Univariate ANOVA).*

Table 3.3.11 LCMRglu response to 8-OH-DPAT in *h*SERT over-expressing mice: non-specific regions

	Male						Female					
	Wild-type			*h*SERT OVR			Wild-type			*h*SERT OVR		
	Saline	8-OH-DPAT	%	Saline	8-OH-DPAT	%	Saline	8-OH-DPAT	%	Saline	8-OH-DPAT	%
Non-specific												
Septal Nucleus	34 ± 3	23 ± 3	**-35****	31 ± 3	21 ± 2	**-30**†	29 ± 2	15 ± 2	**-50*****	18 ± 2	13 ± 2	**-38***†
BNST	27 ± 3	16 ± 2	**-45****	22 ± 2	14 ± 1	**-38***	17 ± 2	10 ± 2	**-40****	11 ± 2	9 ± 1	**-23**
Corpus Callosum	16 ± 1	10 ± 1	**-35**	14 ± 1	9 ± 1	**-35**	11 ± 2	7 ± 1	**-35**	8 ± 1	8 ± 2	**0**
Lateral Habenula	53 ± 5	40 ± 4	**-25***	49 ± 5	35 ± 2	**-29**$^{\#}$	55 ± 4	27 ± 3	**-50*****$^{+}$	36 ± 2	24 ± 3	**-33***$^{\#}$
Mamillary Body	54 ± 4	35 ± 4	**-36****	52 ± 5	27 ± 2	**-47***$^{\#}$	56 ± 3	20 ± 3	**-63*****$^{+}$	38 ± 2	21 ± 2	**-44****$^{\#}$
Periaqueductal Grey	33 ± 3	22 ± 2	**-25****	27 ± 2	17 ± 1	**-37***	23 ± 3	10 ± 2	**-56****	17 ± 2	10 ± 1	**-40***
Inferior Colliculus	73 ± 7	69 ± 9	**-30**	74 ± 10	54 ± 5	**-27**	81 ± 7	44 ± 6	**-46****$^{+}$	54 ± 4	40 ± 4	**-28**
Ventral Tegmental Nucleus	46 ± 4	30 ± 3	**-39*****	42 ± 4	26 ± 2	**-38***	43 ± 3	19 ± 3	**-54****	29 ± 2	20 ± 2	**-33***
Locus Coeruleus	41 ± 4	45 ± 5	**-37***	38 ± 4	25 ± 2	**-36***	38 ± 3	17 ± 3	**-54****	25 ± 2	18 ± 2	**-30**
Nucleus Tractus Solitaris	56 ± 9	25 ± 3	**-53***	50 ± 6	24 ± 1	**-51****	37 ± 4	37 ± 4	**-55***	17 ± 1	15 ± 2	**-19**

*Effect of hSERT over-expression on the LCMRglu response to 8-OH-DPAT in non-specific brain regions. Data shown as mean ± s.e.m and % difference in LCMRglu between 8-OH-DPAT-treated and control (saline-treated) animals of the same gender and genotype. ** denotes p<0.01 and ***denotes p<0.001 significant 8-OH-DPAT effect (within-genotype 2-way ANOVA with Bonferroni correction). +denotes p<0.05 significant gender x 8-OH-DPAT interaction (within-genotype 2-way ANOVA). #denotes p<0.05 significant gender x 8-OH-DPAT x genotype interaction and †denotes p<0.05 significant genotype x 8-OH-DPAT effect in both males and females (Univariate ANOVA). BNST=Bed nucleus of the stria terminalis.*

3.4 Discussion and Conclusions

The distribution and densities of [^{3}H]WAY100,635 binding identified in this study are in strong accordance with those previously reported by other investigators (Fabre et al., 2000a; Preece et al., 2004). We found that [^{3}H]WAY100,635 binding was significantly increased in females as compared to males in the cortex, hippocampus and the raphé. Reports detailing gender differences in cortical 5-HT$_{1A}$ receptor binding in rodents are inconsistent, with some supporting increased 5-HT$_{1A}$ binding (Schiller et al., 2006) while others report no difference (Ferrari et al., 1999; Zhang et al., 1999). Furthermore, gender differences in post-synaptic 5-HT$_{1A}$ binding in the hippocampus display even greater inconsistency with increases (Mendelson and McEwen, 1991), decreases (Li et al., 2000; Schiller et al., 2006) and no difference (Ferrari et al., 1999; Zhang et al., 1999) reported in females as compared to males. Several factors could contribute to the inconsistencies observed between these studies including the use of aged animals, as age-dependent decreases in 5-HT$_{1A}$ binding are stronger in males than in females (Meltzer et al., 2001; Yamaguchi and Yamagata, 1991). Furthermore, lack of appropriate control for the influence of the female reproductive cycle on 5-HT$_{1A}$ binding (Biegon et al., 1980; Uphouse et al., 1986) and the use of different ligands between studies may also contribute to these inconsistencies. To control for the influence of these factors our studies were completed in young animals (4-6 months) and 5-HT$_{1A}$ binding levels were determined in female animals spread across the reproductive cycle. As we used the 5-HT$_{1A}$ antagonist [^{3}H]WAY100,635 in these studies this may explain some of the discrepancies between our data and that reported by others using 5-HT$_{1A}$ agonists, such as [^{3}H]-8-OH-DPAT. As an antagonist [^{3}H]WAY100,635 detects both high and low affinity 5-HT$_{1A}$ binding sites (Gozlan et al., 1995) whereas agonists bind to only high affinity state 5-HT$_{1A}$ receptors directly coupled to their effector G-proteins. This may, in part, account for the significantly increased 5-HT$_{1A}$ binding found in our study in the raphé nuclei of females as compared to males in contrast to others who have found no significant gender difference when using [^{3}H]8-OH-DPAT (Bouali et al., 2003). However, our finding of increased 5-HT$_{1A}$ binding in the raphé of females is likely to represent increased binding at the 5-HT$_{1A}$ autoreceptor which would be consistent with the reported reduced rate of 5-HT neuronal firing (Klink et al., 2002) and enhanced 5-HT$_{1A}$ autoreceptor sensitivity (Bouali et al., 2003; Maswood et al., 1995) in females as compared to males.

In this study 8-OH-DPAT resulted in a generalised depression in cerebral function in all animals, as reflected by widespread significant decreases in LCMRglu. These findings were

similar to previously published reports on the metabolic effect of 8-OH-DPAT on cerebral function in the rat as determined by the quantitative [^{14}C]-2-DG method (Ferrington et al., 2005; Kelly et al., 1988; McBean et al., 1991). However, the significant decreases in LCMRglu observed in our study were more extensive than those previously reported and most likely result from the use of a higher dose of 8-OH-DPAT (10mg.kg^{-1} versus 1mg.kg^{-1}) in this study. This may also account for the fact that we observed no significant increases in the LCMRglu in response to 8-OH-DPAT in contrast to the limited number of increases reported in other studies. In line with the increased 5-HT$_{1A}$ binding in females we also found evidence for increased 5-HT$_{1A}$ functioning, as demonstrated by a significantly enhanced LCMRglu response to 8-OH-DPAT, in a number of brain regions in females as compared to males. Interestingly, 5 of the 6 regions identified as having an increased response to 8-OH-DPAT in females have no detectable levels of 5-HT$_{1A}$ receptor binding. The enhanced metabolic response to 8-OH-DPAT in these regions likely results from the increased functioning of 5-HT$_{1A}$ receptors at sites projecting to these areas as alterations in glucose utilisation primarily reflect alterations in energy requirements at the nerve terminal rather than cell bodies (McCulloch, 1982a). As all of these regions receive dense innervation from multiple brain regions rich in 5-HT$_{1A}$ receptors it is difficult to localise the origin of enhanced 5-HT$_{1A}$ receptor functioning responsible this gender difference. One may speculate on the possible involvement of enhanced 5-HT$_{1A}$ autoreceptor function in females in these responses as electrostimulation of the raphé results in an increased LCMRglu in many of the regions in which this gender difference is observed including the lateral habenula, globus pallidus and mamillary body (Bonvento et al., 1991; Cudennec et al., 1993; Cudennec et al., 1988a). Activation of 5-HT$_{1A}$ autoreceptors could plausibly result in decreased LCMRglu in these regions as a result of decreased raphé 5-HT neuronal activity. Therefore, enhanced 5-HT$_{1A}$ autoreceptor function in females, which is also consistent with our findings in [^{3}H]WAY100,635 binding in the raphé, could contribute to the gender differences in the response to 8-OH-DPAT in these regions. This suggestion is consistent with gender differences in 5-HT$_{1A}$ autoreceptor function reported by others (Bouali et al., 2003; Maswood et al., 1995). However, given the widespread increase in post-synaptic 5-HT$_{1A}$ binding noted in females and the knowledge that each region in which the enhanced response to 8-OH-DPAT was observed in females also receive dense innervation from forebrain sites rich in post-synaptic 5-HT$_{1A}$ receptors it is impossible to rule out their involvement in the sexually dimorphic LCMRglu response to 8-OH-DPAT. While the observed gender differences in post-synaptic 5-HT$_{1A}$ binding observed in this study and by others (Mendelson and McEwen, 1991; Schiller et al., 2006) are suggestive of enhanced post-synaptic 5-HT$_{1A}$

functioning in females no research to date has specifically characterised gender differences in post-synaptic 5-HT_{1A} coupling or function. In future, the utilisation of methodologies such as GTPγS autoradiography and electrophysiological techniques may be able to elucidate the specific localisation of the enhanced 5-HT_{1A} receptor function in females.

Although our data suggest that 5-HT_{1A} function is increased in females it does not allow us to draw conclusion upon the basal level of 5-HT_{1A} receptor activation in females as compared to males. It may be suggested that the enhanced 5-HT_{1A} receptor function present in females may represent an adaptive upregulation of 5-HT_{1A} receptor function in response to the decreased levels of extracellular 5-HT (Gundlah et al., 1998; Jones and Lucki, 2005; Mitsushima et al., 2006), governed in part by the enhanced SERT functioning present in females (study 2), in order to maintain a similar level of 5-HT_{1A} receptor activation to that observed in males. However, we found no significant correlation between the magnitude of the gender difference in SERT binding and that of 5-HT_{1A} receptor binding in a region dependent basis. This suggests that gender differences in SERT function, and so the extracellular 5-HT levels governed by this, may not be the primary mechanism responsible for gender differences in 5-HT_{1A} expression. However, it is also possible that differences in 5-HT_{1A} expression may also not be the primary mechanism by which gender differences in 5-HT_{1A} function are mediated. In the future it will be important to elucidate if gender differences in basal 5-HT_{1A} receptor activity occur as these may contribute to the known gender differences in affective disorders. One possible experiment that may be able to clarify this point would be to investigate the LCMRglu response to 5-HT_{1A} antagonists in animals of both sexes.

In these studies we also found that the life-long increase in SERT functioning in *h*SERT OVR mice decreased post-synaptic 5-HT_{1A} binding levels in a restricted number of hippocampal subfields. In both males and females *h*SERT OVR reduced 5-HT_{1A} binding in the dentate gyrus of the dorsal hippocampus and dentate PO of the ventral hippocampus. However, the impact of altered SERT function on post-synaptic 5-HT_{1A} binding was also found to be greater in males than in females, as evidenced by the significant reduction in 5-HT_{1A} binding in the ventral CA1 and ventral subiculum of males but not females. These findings are not only diametrically opposed to our original hypothesis, that post-synaptic 5-HT_{1A} binding would increase as a result of the increased SERT functioning present in hSERT OVR animals, but also contradicts 5-HT_{1A} binding data in both human (David et al., 2005) and animal (Li et al., 2000) studies showing that a life-long *decrease* in SERT

function *decreases* post-synaptic 5-HT_{1A} binding. However, others have also reported that a life-long decrease in SERT functioning increases post-synaptic 5-HT_{1A} receptor binding in some cortical regions (Lee et al., 2005). Furthermore, the regional distribution of detected alterations in 5-HT_{1A} binding in our study differ from those reported in these previous studies. Indeed, in no region in which 5-HT_{1A} receptor expression was reported to be altered as a result of decreased SERT functioning did we find evidence for a significant alteration in 5-HT_{1A} binding in hSERT OVR mice. These regions included the raphé, amygdala nuclei, the hypothalamus and many cortical regions. In fact the only region in which we found evidence for a significant increase in 5-HT_{1A} binding in hSERT OVR mice was the somatosensory cortex. 5-HT_{1A} binding in this region was not reported to be altered in any of the previous studies, although a linear relationship between genetically determined SERT function and 5-HT_{1A} binding in other cortical regions has been reported (David et al., 2005; Li et al., 2000). Interestingly, this effect was only observed in females suggesting that alterations in 5-HT_{1A} binding in this region were more sensitive in females than in males to alterations in SERT functioning. This finding parallels that of others on the enhanced sensitivity of altered 5-HT_{1A} expression in response to genetically determined SERT function in females as compared to males (as observed in SERT KO mice) (Li et al., 2000).

The decreased 5-HT_{1A} binding found in *h*SERT OVR mice was limited to specific hippocampal subfield whereas previous studies had reported no alteration in hippocampal 5-HT_{1A} binding in response to decreased SERT functioning. However, the studies conducted in humans may have been unable to detect alterations in binding in specific hippocampal subfields due to the low resolution of PET. Indeed, David et al. (2005) reported only on total hippocampal 5-HT_{1A} binding levels which may mean that any significant alteration in a specific hippocampal subfield may have been obscured by a general trend for no alteration in 5-HT_{1A} binding in the hippocampus. However, in agreement with our findings others have reported a trend towards increased total hippocampal 5-HT_{1A} [^{3}H]WAY100,635 binding in animals where SERT function is ablated (SERT KO mice) and have confirmed a significant increase in 5-HT_{1A} protein levels in the hippocampus with antibody binding in these animals (Fabre et al., 2000a). These findings suggest that the regulation of hippocampal 5-HT_{1A} binding by genetically determined SERT functioning may not be directly linked to the regulation of extracellular 5-HT levels by SERT. A possible alternative mechanism may involve the regulation of 5-HT_{1A} receptor binding in the hippocampus by glucocorticoids. A multitude of evidence suggests that 5-HT_{1A} receptor expression and binding in the hippocampus is suppressed by corticosterone (Chalmers et al., 1993; Fernandes et al., 1997;

Neumaier et al., 2000; Takao et al., 1997) and that this mechanism primarily involves activation of the mineralocorticoid receptor (MR) (Meijer et al., 1997; Meijer and Dekloet, 1995). Therefore, it is of great interest that we found MR receptor mRNA levels were increased in the hippocampus of *h*SERT OVR mice (Study 6). This suggests that increased activation of hippocampal MR receptors may be involved in the decreased 5-HT_{1A} binding in the hippocampus of *h*SERT OVR mice. Furthermore, decreased hippocampal 5-HT_{1A} receptor activation in *h*SERT OVR mice may be involved in their reduced anxiety phenotype as the activation of hippocampal 5-HT_{1A} receptors by the local application of 8-OH-DPAT has been shown to have an anxiogenic effect (File et al., 1996). At the present time it is unclear if developmental events related to the level of 5-HT_{1A} activation in the hippocampus of SERT OVR mice may be involved in determining their anxiolytic behavioural phenotype. Evidence from 5-HT_{1A} receptor KO mice suggests that the functional state of forebrain 5-HT_{1A} receptors during the early postnatal period plays a role in determining anxiety-like behaviour (Gross et al., 2002) as the functional reinstatement of these receptors at this time reverses the increased anxiety observed in 5-HT_{1A} receptor KO mice. The exact anatomical localisation of the 5-HT_{1A} receptors responsible for this behavioural rescue, however, is unknown. If hippocampal 5-HT_{1A} receptors are involved in this effect it is plausible that developmental events regulated by hippocampal 5-HT_{1A} receptors, which are decreased in SERT OVR mice, may contribute to their anxiolytic behavioural phenotype. In the future, greater anatomical resolution of 5-HT_{1A} receptor functional reinstatement in 5-HT_{1A} KO mice may provide evidence for the importance of hippocampal 5-HT_{1A} receptors in determining anxiety levels through developmental mechanisms.

In contrast to our findings of altered post-synaptic 5-HT_{1A} binding in *h*SERT OVR mice we found no evidence for altered 5-HT_{1A} autoreceptor binding in these animals. This is also in contrast to reports in which 5-HT_{1A} autoreceptor binding has been found to be decreased in animals where SERT function is knocked-out (Li et al., 2000). However, as the number of 5-HT cell bodies in the raphé of SERT KO mice is reduced this may also contribute to the reduced 5-HT_{1A} autoreceptor levels observed in these animals (Lira et al., 2003b). It is not yet known if 5-HT neuronal number is altered in *h*SERT OVR mice.

In parallel to the finding of decreased 5-HT_{1A} receptor binding in the hippocampus of *h*SERT OVR mice we also found evidence for decreased 5-HT_{1A} functioning in these animals. Again, however, it is difficult to determine the specific localisation of the decreased 5-HT_{1A} receptor functioning that may be responsible for the decreased LCMRglu response to

8-OH-DPAT in *h*SERT OVR mice. Furthermore, the contribution of decreased hippocampal 5-HT_{1A} binding to these functional differences is unclear, especially in light of the fact that alterations in 5-HT_{1A} binding in *h*SERT OVR mice appear to be greater in males than in females whereas the opposite gender effect is noted in 5-HT_{1A}-mediated functional responses. In addition, evidence from SERT KO mice suggests that hippocampal 5-HT_{1A} receptor function is maintained in spite of the reported increase in hippocampal 5-HT_{1A} receptor levels in these animals (Fabre et al., 2000a; La Cour et al., 2001). This suggests that alterations in hippocampal 5-HT_{1A} binding are unlikely to represent the primary mechanisms by which the functional response to 8-OH-DPAT is altered in *h*SERT OVR mice. The localisation and mechanism leading to decreased 5-HT_{1A} functional responses in *h*SERT OVR mice, therefore, remain unclear. However, one suggested mechanism may involve decreased G-protein coupling of the 5-HT_{1A} receptor. However, evidence from SERT KO mice suggests that the G-protein coupling of post-synaptic and pre-synaptic 5-HT_{1A} receptors is not altered in response to genetically determined SERT function. Alternatively, it may be suggested that alterations in 5-HT_{1A} autoreceptor rather than post-synaptic receptor function may be central to the attenuated responses to 8-OH-DPAT in *h*SERT OVR mice. In SERT KO animals decreased 5-HT_{1A} receptor mediated responses can be attributed to the decreased level of 5-HT_{1A} autoreceptor expression in the raphé of SERT KO mice (Li et al., 2000; Li et al., 1999) whereas we found no evidence for altered 5-HT_{1A} receptor binding in the raphé of *h*SERT OVR mice. However, as the level of 5-HT_{1A} receptor binding in our study relates to the total number of 5-HT_{1A} receptors present, rather than those which are directly coupled to G-proteins and in the high affinity state, it is still plausible that the number of functionally coupled 5-HT_{1A} autoreceptors is reduced in *h*SERT OVR mice in the presence of unaltered total 5-HT_{1A} receptor levels. Furthermore, physiological responses linked to the activation of the 5-HT_{1A} autoreceptor have indeed been shown to be decreased in *h*SERT OVR mice (Jennings et al., 2004). The involvement of 5-HT_{1A} autoreceptors in these responses is also consistent with the increased sensitivity of females in the functional adaptation of 5-HT_{1A} autoreceptor function to a life-long decrease in SERT activity (Li et al., 2000). Although it is interesting to speculate on the possible localisation and mechanisms of 5-HT_{1A} receptor alterations that may be involved in the decreased LCMRglu to 8-OH-DPAT in *h*SERT OVR mice it is impossible to make decisive judgements based upon the present data and further research is needed.

Mechanistically, one may suggest that decreased 5-HT_{1A} autoreceptor function in *h*SERT OVR mice may reflect a developmental adaptation to the increased level of SERT expression

present in these animals. Such a decrease in 5-HT_{1A} autoreceptor function would be likely to result in the dis-inhibition of raphé 5-HT neuronal activity as an attempt to maintain normal levels of extracellular 5-HT in these animals in the face of increased 5-HT uptake. However, if this adaptation does occur it is not sufficient to maintain normal extracellular 5-HT levels in these animals (Jennings et al., 2006).

To summarise, we found evidence for enhanced 5-HT_{1A} receptor binding and function in females as compared to males. Surprisingly, we also found that 5-HT_{1A} receptor binding was decreased in the hippocampus of *h*SERT OVR mice and suggest that this may be related to the enhanced MR function present in the hippocampus of *h*SERT OVR mice. In addition we found that the cerebral metabolic response to 8-OH-DPAT was lower in a number of brain regions of *h*SERT OVR mice and that this effect was greater in females than in males. The alterations in 5-HT_{1A} receptor function observed in *h*SERT OVR mice may contribute to the differences noted in constitutive cerebral metabolism in these animals. Furthermore, gender-dependent alterations in 5-HT_{1A} receptor functioning may relate to the noted gender differences of *h*SERT OVR on constitutive cerebral metabolism.

4. Study 4- 5-$HT_{2A/C}$ receptor binding and function in *h*SERT over-expressing mice

4.1 Rationale

5-$HT_{2A/C}$ receptors have a proposed central role in affective psychopathology and the antidepressant response (see section 4.5.1 and 4.5.2). Furthermore, 5-$HT_{2A/C}$ receptors are widely expressed throughout components of the limbic system and play a prominent role in the regulation of 5-HT neurotransmission as both post-synaptic effectors and in the regulation of raphé 5-HT neuronal activity. Evidence from animals in which SERT function is completely ablated (SERT KO mice) suggests that genetically determined SERT expression may regulate both 5-$HT_{2A/C}$ receptor expression and function. For example, 5-HT_{2A} and 5-HT_{2C} receptor binding has been found to be significantly *increased* in some brain regions of SERT KO mice (Li et al., 2003; Rioux et al., 1999). However, the ability of altered SERT function to regulate 5-HT_{2A} expression appears to be brain region-dependent as 5-HT_{2A} binding was also reported to be significantly *decreased* in other brain areas. In addition, while some hormonal responses mediated by hypothalamic 5-$HT_{2A/C}$ receptors suggest enhanced functioning of these receptors in SERT KO mice (Li et al., 2003) the function of 5-$HT_{2A/C}$ receptors appears to be attenuated in these animals in most other brain regions (Qu et al., 2005). These data suggest that the regulation of 5-$HT_{2A/C}$ receptors by genetically determined SERT function is complicated and occurs in a brain region dependent fashion. The finding that 5-$HT_{2A/C}$ receptor binding is increased in SERT KO mice appears paradoxical to the observation that synaptic 5-HT levels are elevated in these animals. This suggests that the regulation of extracellular 5-HT by SERT functioning may not be the primary mechanism by which SERT regulates 5-$HT_{2A/C}$ receptor expression and that other developmental events may be important. However, the functional desensitisation of 5-$HT_{2A/C}$ receptors in SERT KO animals is consistent with the increased levels of synaptic 5-HT in these animals.

In order to further investigate how genetically determined alterations in SERT may regulate 5-$HT_{2A/C}$ receptor expression and function here we investigate 5-HT_{2A} and 5-HT_{2C} binding in *h*SERT OVR animals using [^{3}H]ketanserin and [^{3}H]mesulergine ligand binding autoradiography, respectively, and investigate the LCMRglu response to DOI in order to characterise 5-$HT_{2A/C}$ receptor function. On the basis of data obtained in SERT KO animals

we hypothesis that 5-$HT_{2A/C}$ receptor binding will be altered in a brain region specific manner and that 5-$HT_{2A/C}$ receptor function will be increased in *h*SERT OVR animals. As our previous data suggest that the ability of *h*SERT OVR to alter cerebral and 5-HT_{1A} receptor functioning is modulated by gender we also hypothesise that the ability of *h*SERT OVR to influence 5-$HT_{2A/C}$ binding and function will also be greater in females than in males.

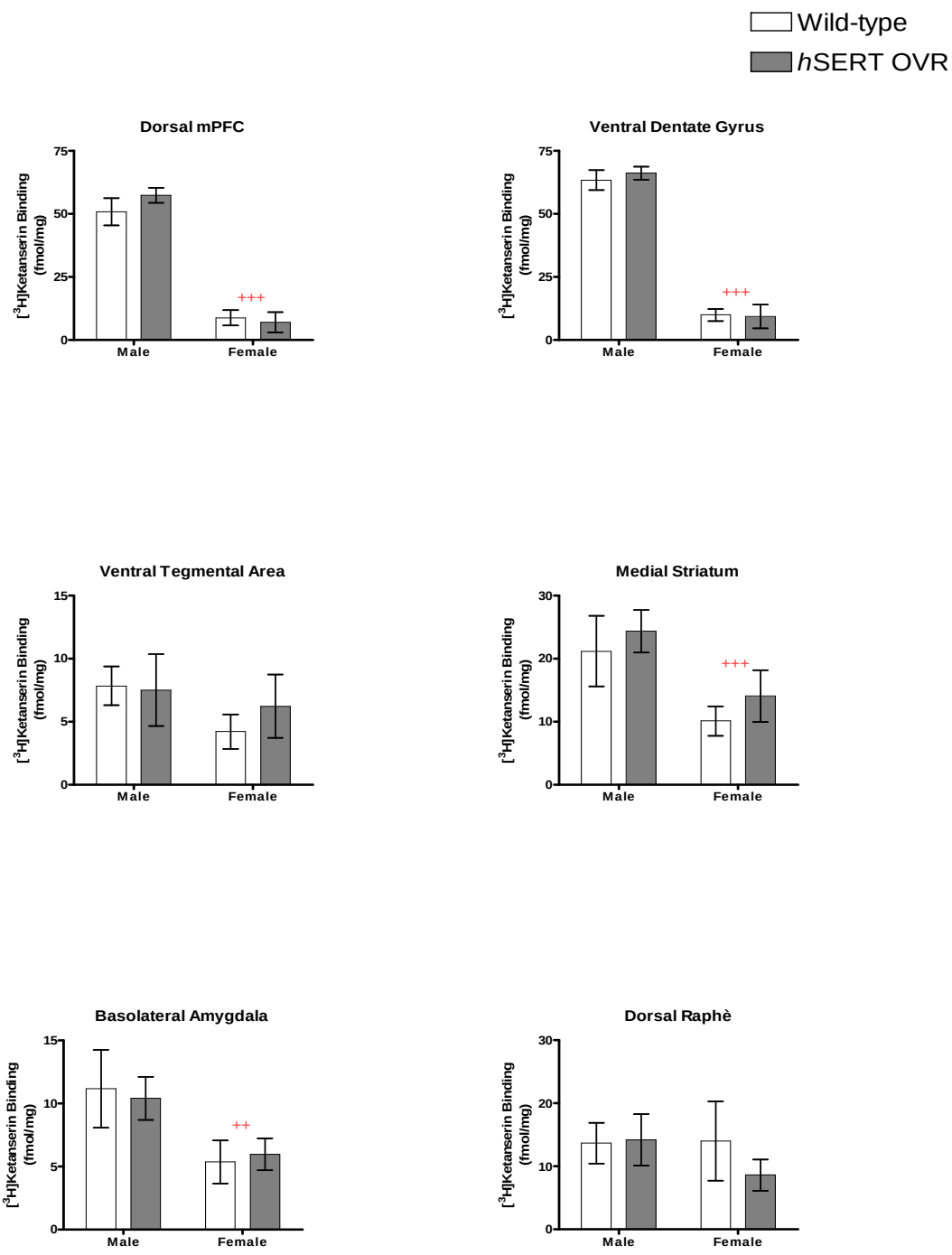
4.2 [^{3}H]Ketanserin Binding

[^{3}H]Ketanserin binding levels were high in the claustrum, endopriform nucleus, orbitofrontal and frontal cortex. Medium levels of binding were detected in many other cortical regions and the ventral dentate gyrus of the hippocampus. Lower levels of binding were detected in the other hippocampal subfields, the striatum and raphé nuclei. The distribution and relative densities of [^{3}H]ketanserin binding in this study is consistent with that reported by others (Ciccocioppo et al., 1999; Pazos et al., 1985; Preece et al., 2004).

[^{3}H]Ketanserin binding tended to be lower in females than in males in all brain regions and was significantly lower in 27 of the 49 brain areas analysed. This effect was observed in all cortical brain regions (range -63% to -103% lower in females), many subfields of the hippocampus (range -46% to -123% lower in females) and multiple hypothalamic nuclei (range -89% to -91% lower in females). Figures 3.4.1 and 3.4.2 show representative brain regions in which [^{3}H]Ketanserin binding was influenced by gender.

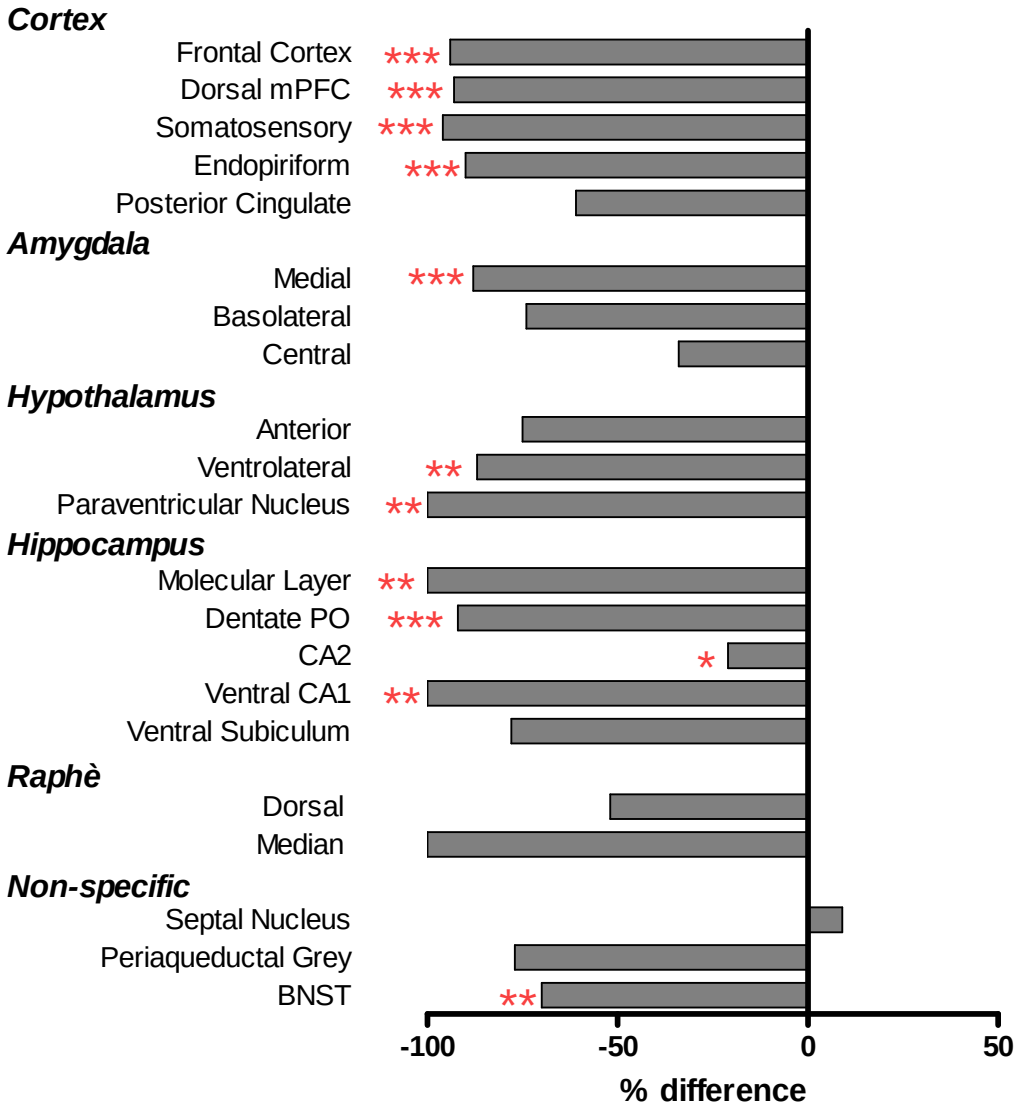
There was no evidence for altered [^{3}H]ketanserin binding in *h*SERT OVR mice in comparison to Wt animals in any of the brain regions analysed. The was also no evidence for a significant gender x *h*SERT OVR interaction on [^{3}H]ketanserin binding in any brain region. Detailed data on [^{3}H]Ketanserin binding are shown in Tables 3.4.1 to 3.4.5.

Figure 3.4.1 [^{3}H]Ketanserin binding in *h*SERT over-expressing mice



[^{3}H]Ketanserin binding in 6 representative brain regions in hSERT OVR and Wt mice of both genders. Data shown as mean ± s.e.m and were analysed using 2-way ANOVA. Y axis values differ between brain region in accordance with maximum binding value. $^{++}$denotes p<0.01, $^{+++}$denotes p<0.01 significant gender effect. [^{3}H]Ketanserin binding is significantly lower in females than in males in a number of brain regions (e.g. dorsal mPFC and basolateral amygdale) but is not altered by hSERT over-expression in animals of either gender.

Figure 3.4.2 Gender differences in [^{3}H]Ketanserin binding



*Gender differences in [^{3}H]Ketanserin binding in a representative selection of diverse brain areas. Data shown as % difference in binding between male and female animals with data from both Wt and hSERT OVR mice. *denotes p<0.05, **denotes p<0.01 and ***denotes P<0.01 significant gender effect (2-way ANOVA). [^{3}H]Ketanserin binding was significantly decreased in females as compared to males.*

Table 3.4.1 [^{3}H]Ketanserin binding in hSERT over-expressing mice: cortical regions

	Male						Female					
	Wild-type			*h*SERT OVR			Wild-type			*h*SERT OVR		
	Mean		s.e.m	Mean		s.e.m	Mean		s.e.m	Mean		s.e.m
Cortex												
Orbitofrontal	76.1	±	3.9	80.6	±	3.4	11.2***	±	2.1	13.2	±	3.0
Frontal	82.5	±	3.8	84.4	±	3.6	10.3***	±	1.8	16.5	±	2.0
Anterior Cingulate	34.9	±	7.2	35.5	±	3.8	4.0***	±	1.5	9.7	±	1.3
Dorsal medial Prefrontal	50.3	±	5.5	57.3	±	3.7	8.8***	±	2.1	7.0	±	2.9
Ventral medial Prefrontal	19.9	±	3.7	27.3	±	3.4	7.2***	±	2.5	8.2	±	3.2
Somatosensory	57.8	±	4.0	63.8	±	4.2	2.8***	±	1.5	8.5	±	1.3
Frontal Motor Cortex (Layer V)	56.9	±	6.8	56.1	±	5.2	9.9***	±	1.8	11.2	±	1.3
Frontal Motor Cortex (Layer VI)	48.1	±	6.2	54.0	±	3.6	11.5***	±	1.8	11.2	±	0.9
Temperoparietal	41.8	±	3.5	42.1	±	2.4	8.6***	±	1.9	13.3	±	3.0
Posterior Cingulate	9.0	±	2.5	9.3	±	2.1	2.8	±	1.8	7.8	±	2.3
Entorhinal	58.0	±	3.2	60.8	±	2.3	6.7***	±	2.1	7.6	±	2.3
Endopiriform	65.0	±	2.3	70.2	±	2.2	5.6***	±	1.4	11.0	±	1.6

*Effect of hSERT over-expression on [^{3}H]Ketanserin binding (fmol/mg) in cortical and basal ganglia regions. Data are expressed as mean ± s.e.m and were analysed using 2-way ANOVA. *denotes p<0.05, **denotes p<0.01, ***denotes p<0.001 significant gender difference (2-way ANOVA).*

Table 3.4.2 [^{3}H]Ketanserin binding in *h*SERT over-expressing mice: basal ganglia

	Male						Female					
	Wild-type			*h*SERT OVR			Wild-type			*h*SERT OVR		
	Mean		s.e.m	Mean		s.e.m	Mean		s.e.m	Mean		s.e.m
Basal Ganglia												
Medial Striatum	21.2	±	5.7	24.4	±	3.8	10.1*	±	1.7	14.1	±	2.9
Lateral Striatum	11.4	±	2.8	6.8	±	3.3	9.4	±	1.8	11.4	±	2.1
Globus Pallidus	3.7	±	1.7	6.0	±	2.3	0	±	0	1.9	±	1.1
Substantia Nigra pars Reticulata	4.8	±	2.6	5.0	±	2.1	12.2	±	2.2	9.0	±	3.5
Substantia Nigra pars Compacta	5.3	±	2.3	2.8	±	1.9	10.9	±	1.5	7.7	±	3.7

*Effect of hSERT over-expression on [^{3}H]Ketanserin binding (fmol/mg) in cortical and basal ganglia regions. Data are expressed as mean ± s.e.m and were analysed using 2-way ANOVA. *denotes p<0.05, **denotes p<0.01, ***denotes p<0.001 significant gender difference (2-way ANOVA).*

Table 3.4.3 [^{3}H]Ketanserin binding in *h*SERT over-expressing mice: amygdala, thalamic and hypothalamic regions

	Male						Female					
	Wild-type			*h*SERT OVR			Wild-type			*h*SERT OVR		
	Mean		s.e.m	Mean		s.e.m	Mean		s.e.m	Mean		s.e.m
Amygdala												
Medial	21.4	±	6.5	24.3	±	2.6	4.8***	±	1.6	7.9	±	3.5
Basolateral	11.3	±	3.1	12.9	±	3.8	3.5	±	1.5	5.9	±	0.8
Central	6.2	±	2.7	6.4	±	1.8	3.8	±	0.6	8.6	±	1.4
Thalamic Nuclei												
Anterior	0.0	±	2.0	5.7	±	1.1	0.2	±	1.4	5.4	±	1.4
Mediodorsal	5.6	±	2.0	4.8	±	2.5	2.1	±	1.7	6.6	±	2.2
Venterolateral	4.9	±	2.3	3.2	±	2.1	3.7	±	1.1	6.9	±	2.3
Hypothalamic Nuclei												
Anterior	8.6	±	1.6	11.3	±	2.2	3.0	±	1.2	2.9	±	1.2
Paraventricular	8.5	±	3.1	11.6	±	1.2	0.1**	±	1.3	4.0	±	2.0
Ventrolateral	11.9	±	1.7	11.8	±	1.9	4.2**	±	1.4	4.9	±	2.0
Ventromedial	20.4	±	5.1	18.9	±	7.3	1.3*	±	1.5	4.0	±	1.8

*Effect of hSERT over-expression on [^{3}H]Ketanserin binding (fmol/mg) in amygdala, thalamic and hypothalamic regions. Data are expressed as mean ± s.e.m and were analysed using 2-way ANOVA. *denotes p<0.05, **denotes p<0.01, ***denotes p<0.001 significant gender difference.*

Table 3.4.4 [^{3}H]Ketanserin binding in *h*SERT over-expressing mice: hippocampal regions

	Male						Female					
	Wild-type			*h*SERT OVR			Wild-type			*h*SERT OVR		
	Mean		s.e.m	Mean		s.e.m	Mean		s.e.m	Mean		s.e.m
Dorsal Hippocampus												
CA1	13.9	±	3.5	17.4	±	1.2	9.5**	±	1.3	7.1	±	2.5
CA2	12.2	±	1.2	13.5	±	0.9	6.7	±	2.6	7.2	±	3.2
CA3	7.5	±	1.9	9.1	±	1.4	6.6	±	1.4	9.2	±	2.6
DG	7.5	±	3.6	5.9	±	2.5	3.9	±	2.0	4.2	±	1.0
Ventral Hippocampus												
Molecular Layer	15.1	±	3.0	14.5	±	1.7	5.7**	±	1.8	5.8	±	0.6
Dorsal Subiculum	12.3	±	1.9	8.8	±	1.9	8.0	±	1.9	7.7	±	2.5
Dentate PO	21.6	±	3.3	24.8	±	2.9	8.8***	±	1.1	7.0	±	2.2
Dorsal CA1	17.0	±	2.0	17.6	±	1.8	6.2***	±	0.7	4.4	±	1.9
CA2	11.7	±	4.3	16.3	±	1.7	6.2*	±	1.1	7.4	±	1.9
Ventral CA1	13.1	±	1.9	11.5	±	2.5	6.5**	±	1.0	4.4	±	1.3
Ventral DG	61.9	±	4.6	66.4	±	2.7	9.9***	±	1.7	8.2	±	3.5
Ventral Subiculum	7.7	±	1.9	8.4	±	2.3	6.4	±	0.9	7.0	±	1.7
CA3	13.2	±	2.3	11.5	±	1.6	9.6*	±	1.4	5.3	±	1.0

*Effect of hSERT over-expression on [^{3}H]Ketanserin binding (fmol/mg) hippocampal regions. Data are expressed as mean ± s.e.m and were analysed using 2-way ANOVA. *denotes p<0.05, **denotes p<0.01, ***denotes p<0.001 significant gender difference.*

Table 3.4.5 [^{3}H]Ketanserin binding in hSERT over-expressing mice: raphé, mesocorticolimbic and non-specific regions

	Male						Female					
	Wild-type			hSERT OVR			Wild-type			hSERT OVR		
	Mean		s.e.m	Mean		s.e.m	Mean		s.e.m	Mean		s.e.m
Raphé												
Dorsal	13.6	±	3.5	14.1	±	4.3	8.1	±	1.6	8.6	±	1.6
Median	9.7	±	2.6	8.1	±	1.6	6.5	±	1.1	8.5	±	1.6
Mesocorticolimbic System												
Ventral Tegmental Area	7.9	±	1.6	7.5	±	2.8	1.2	±	1.1	3.8	±	1.3
Nucleus Accumbens	22.8	±	5.2	34.8	±	5.9	9.7***	±	1.2	11.8	±	2.3
Non-specific												
Claustrum	100.6	±	8.5	108.8	±	4.6	7.6***	±	1.6	13.8	±	3.0
Septal Nucleus	5.7	±	1.6	2.7	±	3.8	4.7	±	2.1	4.3	±	1.5
Bed Nucleus of the Stria Terminalis	11.1	±	2.5	10.7	±	2.0	5.9**	±	1.9	3.2	±	0.9
Lateral Habenula	3.0	±	1.4	3.6	±	2.6	1.5	±	1.1	4.0	±	1.9
Periaqueductal Grey	8.0	±	2.7	9.5	±	2.9	5.6	±	1.8	2.6	±	1.6
Inferior Colliculus	6.7	±	1.1	8.2	±	1.6	3.7	±	1.0	7.7	±	2.2

*Effect of hSERT over-expression on [^{3}H]Ketanserin binding (fmol/mg) raphé, mesocorticolimbic and non-specific brain regions. Data are expressed as mean ± s.e.m and were analysed using 2-way ANOVA. *denotes p<0.05, **denotes p<0.01, ***denotes p<0.001 significant gender difference.*

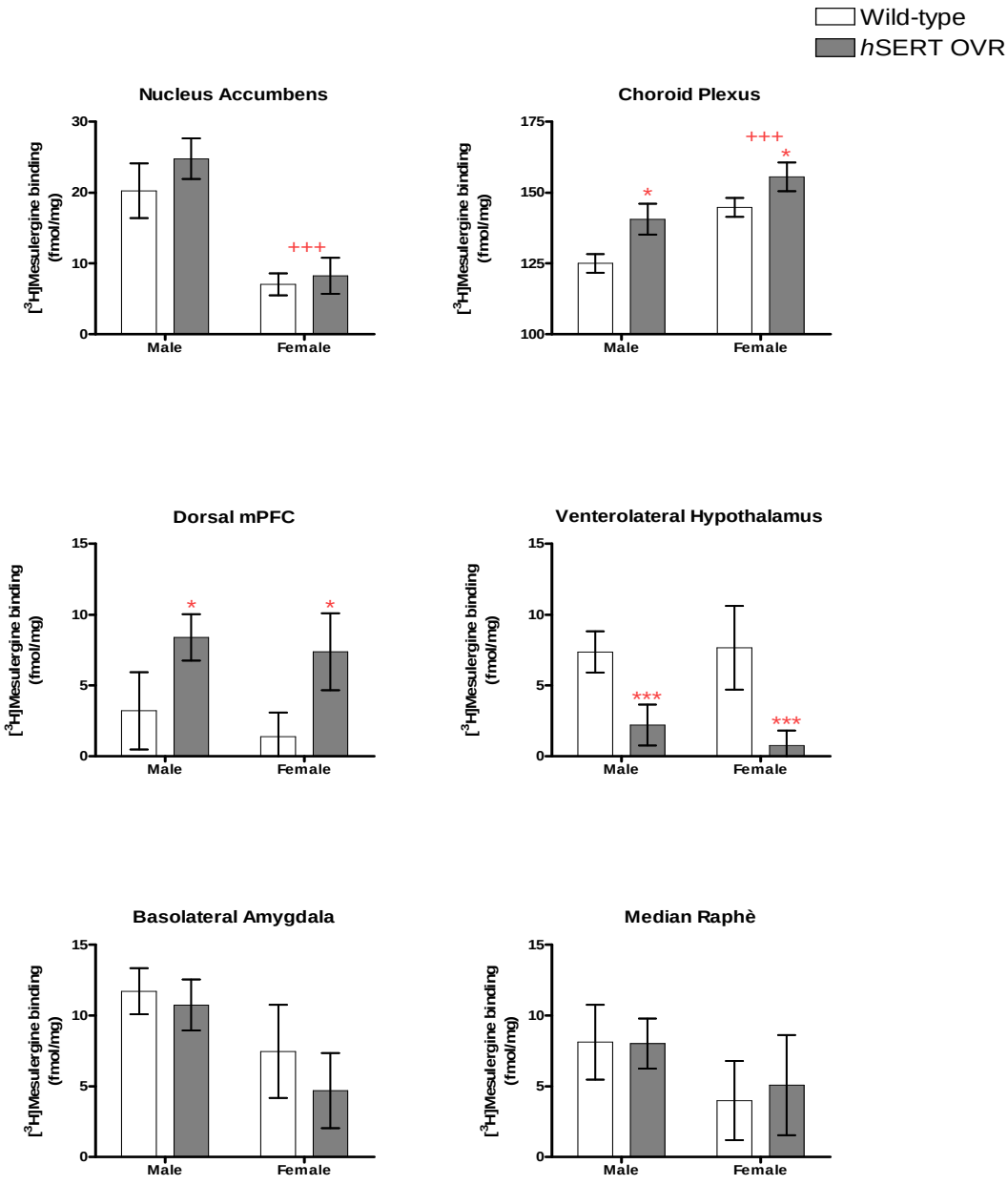
4.3 [^{3}H]Mesulergine Binding

[^{3}H]Mesulergine binding levels were highest in the choroid plexus and nucleus accumbens, whereas medium levels of were detected in the amygdala nuclei, raphé and components of the basal ganglia. Low levels of binding were detected in specific hippocampal subfields and cortical regions. The distribution and density of [^{3}H]mesulergine binding was with consistent with that previously reported (Kuoppamaki et al., 1994; Laakso et al., 1996; Li et al., 2003).

In the majority of brain regions [^{3}H]mesulergine binding tended to lower in females than in males. However, this effect was significant only in the nucleus accumbens (66% lower binding in females). In contrast [^{3}H]mesulergine binding was found to be significantly increased in the choroid plexus (+13%) of females as compared to males. See Figures 3.4.3 and 3.4.4 for gender differences in [^{3}H]mesulergine binding in a number of representative brain regions.

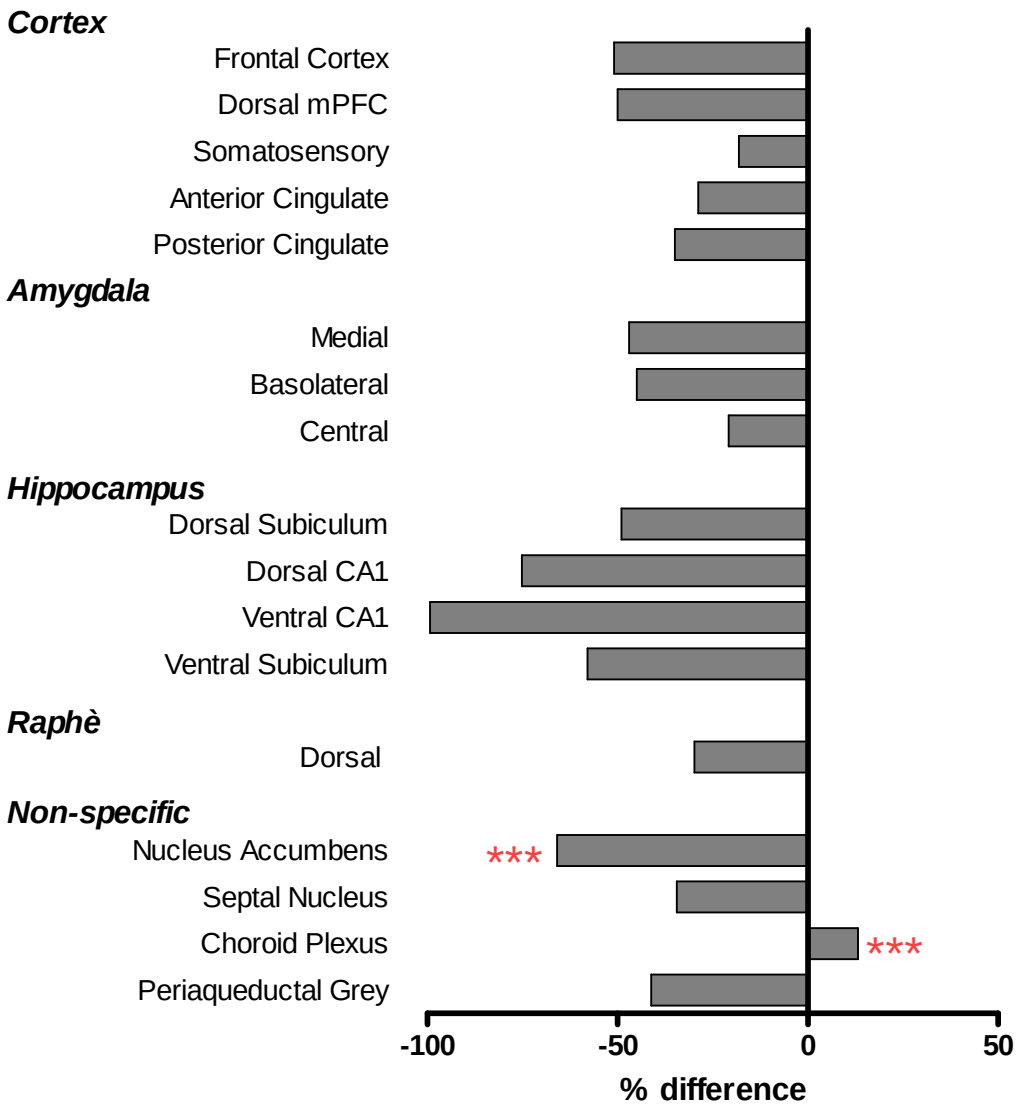
*h*SERT OVR significantly *increased* [^{3}H]mesulergine binding in both the choroids plexus (mean +16%) and dorsal mPFC (mean +453%) but significantly *decreased* binding in the venterolateral hypothalamus (mean -100%) (Figure 3.4.3). In all other brain regions binding was unaltered in *h*SERT OVR mice in comparison to their Wt littermates. In addition, there was no evidence for an interaction between gender and *h*SERT OVR on [^{3}H]mesulergine binding in any brain region, suggesting that gender does not influence the ability of *h*SERT OVR to alter [^{3}H]mesulergine binding. Detailed data on [^{3}H]mesulergine binding are shown in Tables 3.4.6 to 3.4.10.

Figure 3.4.3 [^{3}H]Mesulergine binding in *h*SERT over-expressing mice



*[^{3}H]Mesulergine binding in 6 representative brain regions in male and female hSERT OVR and Wt mice. Data shown as mean ± s.e.m and were analysed using 2-way ANOVA. *denotes p<0.05, ***denotes p<0.01 significant hSERT OVR effect. $^{+++}$denotes p<0.01 significant gender effect. [^{3}H]mesulergine binding was significantly lower in the nucleus accumbens but significantly higher in the choroids plexus in females as compared to males. [^{3}H]Mesulergine binding was significantly increased in the dorsal mPFC and choroids plexus but decreased in the ventrolateral hypothalamus of hSERT OVR mice of both genders.*

Figure 3.4.4 Gender differences in [^{3}H]Mesulergine binding



*Gender differences in [^{3}H]Mesulergine binding in selected brain regions from diverse functional systems. Data shown as percentage difference between female relative to male mice from both genotypes. % difference between genders appears to be great but non-significant in many regions (e.g. ventral CA1) due to low level of binding and a relatively high variance in these areas. Whereas, a relatively small % difference is significant in the choroids plexus as binding is particularly high in this region. ***denotes p<0.001 significant gender difference (2-way ANOVA). [^{3}H]mesulergine binding was significantly lower in the nucleus accumbens of females as compared to males whereas in the choroids plexus binding was significantly higher in females.*

Table 3.4.6 [^{3}H]Mesulergine binding in hSERT over-expressing mice: cortical regions

	Male						Female					
	Wild-type			*h*SERT OVR			Wild-type			*h*SERT OVR		
	Mean		s.e.m	Mean		s.e.m	Mean		s.e.m	Mean		s.e.m
Cortex												
Orbitofrontal	2.43	±	2.37	4.82	±	2.29	1.00	±	2.53	1.30	±	1.58
Frontal	3.91	±	1.91	4.22	±	1.96	3.44	±	2.37	0.43	±	2.43
Anterior Cingulate	8.63	±	1.07	9.91	±	2.58	6.28	±	3.13	3.86	±	1.73
Dorsal medial Prefrontal	1.91	±	2.81	8.39	±	1.64$^+$	0.93	±	1.62	6.17	±	2.62$^+$
Ventral medial Prefrontal	1.75	±	2.85	7.34	±	1.32	3.47	±	1.02	5.11	±	2.43
Somatosensory	4.28	±	2.52	5.50	±	2.61	5.70	±	1.67	1.69	±	2.11
Temperoparietal	1.33	±	1.74	0.85	±	0.49	0.25	±	3.04	0.6	±	3.15
Posterior Cingulate	10.62	±	1.11	10.03	±	1.90	10.15	±	3.29	6.80	±	1.80
Piriform	10.01	±	1.62	12.68	±	3.67	6.44	±	1.74	10.38	±	3.37
Entorhinal	6.95	±	1.96	2.14	±	3.74	2.58	±	2.31	6.37	±	3.41

Effect of hSERT over-expression on [^{3}H]Mesulergine binding (fmol/mg)in cortical and basal ganglia regions. Data are expressed as mean ± s.e.m and were analysed using 2-way ANOVA.$^+$denotes p<0.05 significant genotype effect.

Table 3.4.7 [^{3}H]Mesulergine binding in hSERT over-expressing mice: basal ganglia regions

	Male						Female					
	Wild-type			*h*SERT OVR			Wild-type			*h*SERT OVR		
	Mean		s.e.m	Mean		s.e.m	Mean		s.e.m	Mean		s.e.m
Basal Ganglia												
Medial Striatum	9.16	±	2.89	11.44	±	3.88	5.85	±	2.95	6.13	±	2.53
Lateral Striatum	9.71	±	3.42	8.94	±	3.01	2.83	±	3.07	2.38	±	2.64
Globus Pallidus	3.08	±	1.62	8.30	±	1.59	1.79	±	2.41	0.65	±	2.40
Subthalamic Nucleus	7.66	±	1.34	7.23	±	1.29	10.09	±	3.09	0.74	±	1.38
Substantia Nigra pars Reticulata	8.83	±	2.17	10.44	±	2.01	4.29	±	4.41	4.70	±	2.60
Substantia Nigra pars Compacta	7.4	±	2.20	8.13	±	1.52	6.98	±	4.03	5.96	±	2.90

Effect of hSERT over-expression on [^{3}H]Mesulergine binding (fmol/mg)in cortical and basal ganglia regions. Data are expressed as mean ± s.e.m and were analysed using 2-way ANOVA $^+$denotes p<0.05 significant genotype effect.

Table 3.4.8 [^{3}H]Mesulergine binding in *h*SERT over-expressing mice: amygdala, thalamic and hypothalamic regions

	Male						Female					
	Wild-type			*h*SERT OVR			Wild-type			*h*SERT OVR		
	Mean		s.e.m	Mean		s.e.m	Mean		s.e.m	Mean		s.e.m
Amygdala												
Medial	13.89	±	1.89	9.82	±	1.30	7.93	±	3.43	8.19	±	2.74
Basolateral	11.70	±	1.62	10.74	±	1.80	9.06	±	3.21	4.69	±	2.54
Central	10.67	±	1.92	7.52	±	1.09^{+}	10.57	±	3.72	6.65	±	2.16^{+}
Thalamic Nuclei												
Anterior	1.86	±	3.09	3.31	±	1.99	1.52	±	3.03	1.73	±	2.48
Mediodorsal	7.54	±	2.38	8.43	±	1.86	8.20	±	3.40	1.49	±	1.61
Venterolateral	1.41	±	1.49	2.42	±	1.14	2.79	±	2.93	-3.32	±	1.55
Hypothalamic Nuclei												
Anterior	8.21	±	2.35	7.51	±	1.17	6.97	±	3.42	5.79	±	2.46
Venterolateral	7.37	±	1.46	2.20	±	1.34^{+++}	7.66	±	2.83	0.76	±	0.97^{+++}

Effect of hSERT over-expression on [^{3}H]Mesulergine binding (fmol/mg)in amygdala ,thalamic and hypothalamic regions. Data are expressed as mean ± s.e.m and were analysed using 2-way ANOVA.$^{+}$denotes p<0.05, +++denotes p<0.001 significant genotype effect.

Table 3.4.9 [^{3}H]Mesulergine binding in *h*SERT over-expressing mice: hippocampal regions

	Male						Female					
	Wild-type			*h*SERT OVR			Wild-type			*h*SERT OVR		
	Mean		s.e.m	Mean		s.e.m	Mean		s.e.m	Mean		s.e.m
Dorsal Hippocampus												
CA1	3.54	±	2.17	0.74	±	0.80	1.31	±	3.54	0	±	0
CA2	3.51	±	1.74	0	±	0	0	±	0	0	±	0
CA3	3.94	±	1.67	0	±	0	1.06	±	2.58	0	±	0
DG	1.35	±	1.73	0.13	±	0.84	0.60	±	3.50	0	±	0
Ventral Hippocampus												
Molecular Layer	5.27	±	2.39	3.64	±	1.46	0	±	0	4.34	±	1.99
Dorsal Subiculum	5.86	±	1.86	6.00	±	2.12	0.12	±	2.68	2.75	±	2.49
Dentate PO	2.60	±	1.93	0.77	±	2.19	0.14	±	1.42	2.47	±	2.74
Dorsal CA1	4.42	±	2.17	4.35	±	2.19	0	±	0	2.15	±	2.26
CA2	2.96	±	2.23	6.36	±	1.06	0	±	0	0.82	±	3.13
Ventral CA1	4.19	±	1.90	8.02	±	1.82	0	±	0	3.73	±	2.97
Ventral Suiculum	7.70	±	1.49	12.94	±	1.28	1.31	±	2.15	7.92	±	3.24
CA3	2.27	±	1.65	0.42	±	1.62	0	±	0	5.53	±	2.11

Effect of hSERT over-expression on [^{3}H]Mesulergine binding (fmol/mg) in hippocampal brain regions. Data are expressed as mean ± s.e.m and were analysed using 2-way ANOVA.

Table 3.4.10 [^{3}H]Mesulergine binding in *h*SERT over-expressing mice: raphé, mesocorticolimbic and non-specific regions

	Male						Female					
	Wild-type			hSERT OVR			Wild-type			hSERT OVR		
	Mean		s.e.m	Mean		s.e.m	Mean		s.e.m	Mean		s.e.m
Raphé												
Dorsal	8.90	±	1.94	5.98	±	1.97	3.79	±	2.16	3.71	±	2.77
Median	8.12	±	2.65	8.01	±	1.78	5.72	±	2.30	5.47	±	2.60
Paramedian	5.80	±	1.86	8.72	±	1.87	3.79	±	3.45	1.81	±	2.79
Mesocorticolimbic System												
Ventral Tegmental Area	5.58	±	0.96	5.54	±	3.02	1.40	±	3.62	2.44	±	1.60
Nucleus Accumbens	20.25	±	3.86	24.78	±	2.88	7.05***	±	1.49	9.74	±	2.19
Non-specific												
Choroid Plexus	125.99	±	3.27	141.60	±	5.49^{++}	145***	±	3.34	155.57	±	5.06^{++}
Septal Nucleus	10.27	±	3.88	12.04	±	4.45	7.94	±	2.49	3.00	±	2.18
Bed Nucleus of the Stria Terminalis	6.80	±	2.33	4.97	±	2.54	7.28	±	2.59	3.98	±	2.14
Lateral Habenula	5.01	±	1.98	7.56	±	2.83	5.33	±	3.15	0	±	0
Periaqueductal Grey	10.94	±	1.70	8.56	±	1.85	5.77	±	2.77	5.56	±	2.35
Inferior Colliculus	2.90	±	1.93	4.94	±	1.96	0	±	0	2.56	±	2.09

*Effect of hSERT over-expression on [^{3}H]Mesulergine binding (fmol/mg) in raphé, mesocorticolimbic and non-specific brain regions. Data are expressed as mean ± s.e.m and were analysed using 2-way ANOVA.***denotes p<0.05 significant gender effect ++denotes p<0.01 significant genotype effect.*

4.4 LCMRglu

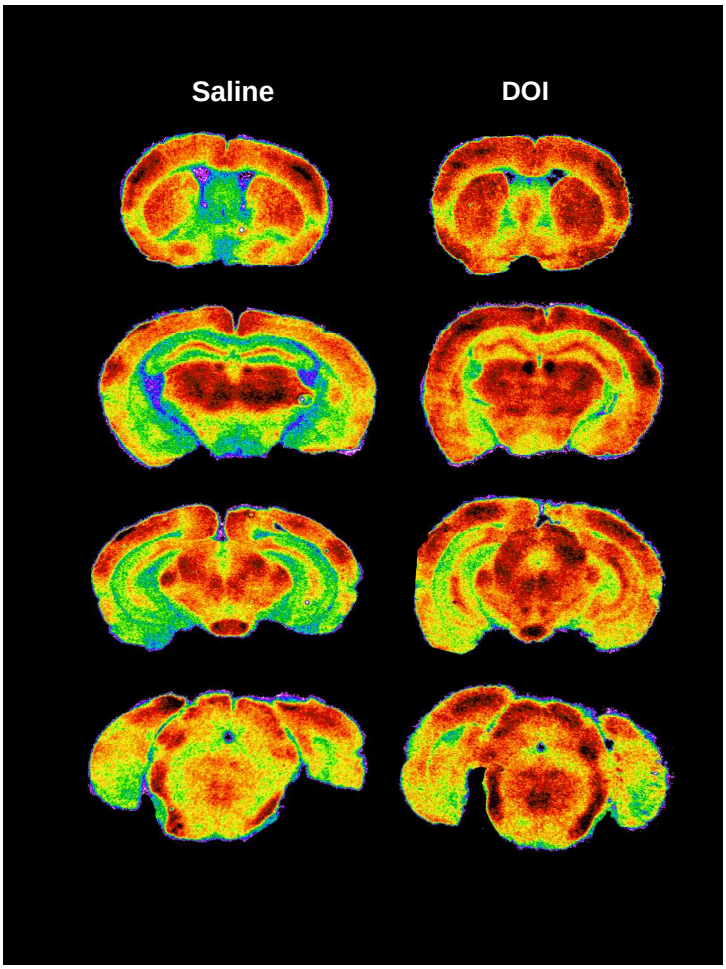
In Wt females there was no evidence for a significant LCMRglu response to DOI in any brain region. In contrast, DOI resulted in 3 significant increases in LCMRglu in Wt males. These were present in the lateral habenula (+52%), ventral tegmental area (+37%) and mamillary body (+40%). This suggested that females were less sensitive to DOI, a finding that was replicated in *h*SERT OVR mice. In addition the LCMRglu response to DOI was both more pronounced and prevalent in *h*SERT OVR as compared to Wt animals. In *h*SERT OVR females DOI significantly increased LCMRglu in 3 regions, the septal nucleus (+34%), the BNST (+61%) and the lateral habenula (+50%). Whereas, in *h*SERT OVR males DOI produced significant increases in LCMRglu in 32 of the 47 regions analysed. The most marked responses in *h*SERT OVR males were observed in the lateral habenula (+124%), the raphé (median, +76%) and in components of the mesocorticolimbic system (nucleus accumbens, +74%; VTA +50%). Increases were also prevalent in component of the basal ganglia (medial striatum, +41%; SNR/SNC +54%) and the hippocampus (molecular layer +40%, ventral subiculum +43%) of *h*SERT OVR males.

Evidence from both genotypes suggested that the response to DOI was lower in females as compared to males. Indeed, a significant effect of gender on the LCMRglu response to DOI was identified in 11 of the 47 brain regions analysed. These included components of the mesocorticolimbic system (nucleus accumbens: female +2%, male +36%; VTA: female +1%, male +44%), all raphè regions (median: female +16%, male +43%) and the lateral habenula (female +22%, male +88%). Figures 3.2.6 and 3.4.7 show representative brain regions in which the LCMRglu response to DOI is greater in males than in females.

In both genders *h*SERT OVR increased the LCMRglu response to DOI with a significant effect found in 20 of the 47 brain regions analysed. This was particularly noted in the raphè (median: males +65%, females +14% increase response in *h*SERT OVR), hippocampus (molecular layer: males +35%, females +36% increase in *h*SERT OVR), components of the motor system (SNR male, +47%, females +46% in *h*SERT) and the lateral habenula (males +73%, females +55% increase response in *h*SERT OVR). Figures 3.4.6, 3.4.8 and 3.4.9 show representative brain regions in which the LCMRglu response to DOI is altered by *h*SERT over-expression.

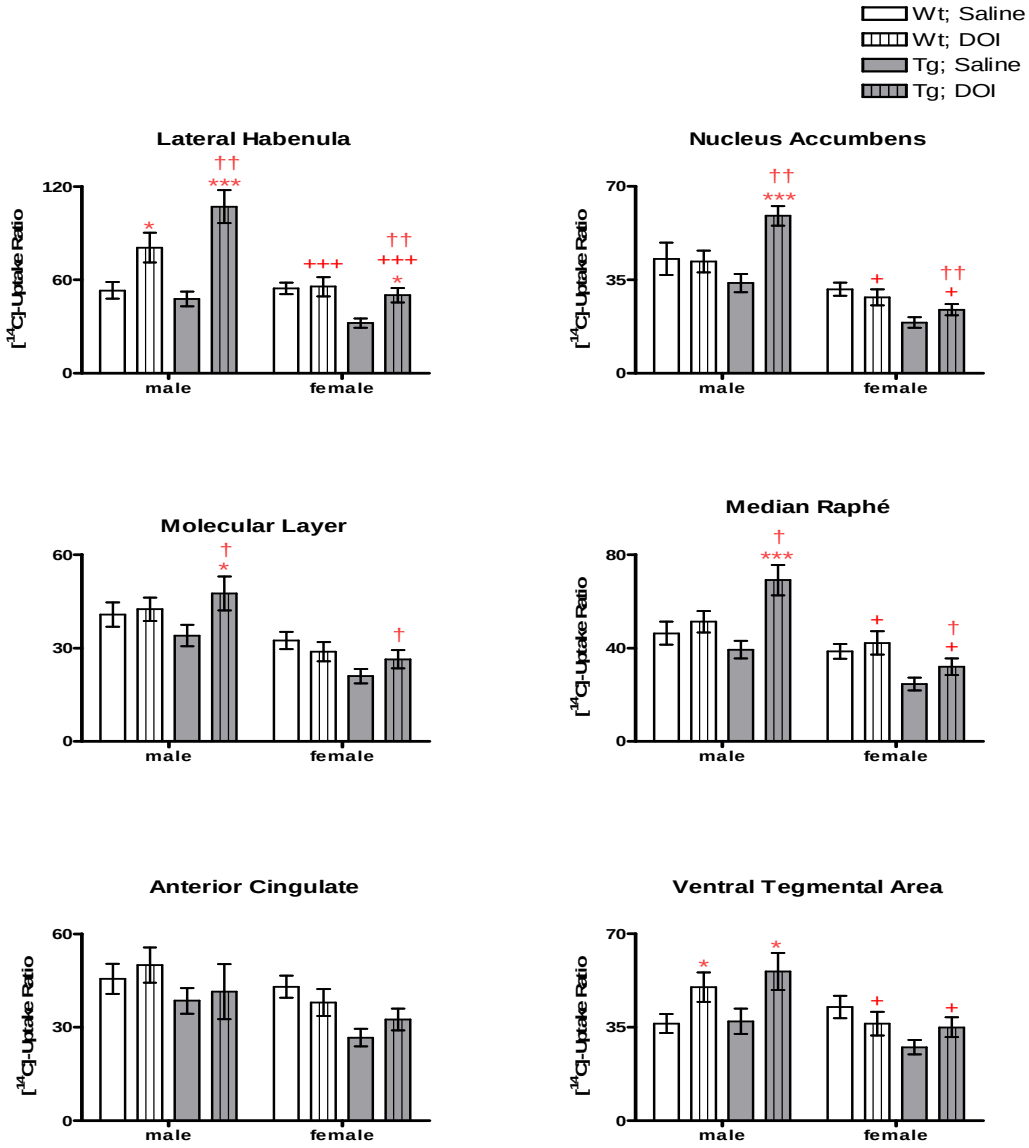
Despite the observation that the enhancement of the response to DOI by *h*SERT OVR tended to be greater in males than in females there was no evidence for a significant gender x *h*SERT OVR x DOI interaction in any brain region. Detailed data on the effects of *h*SERT over-expression on the LCMRglu response to DOI are shown in Tables 3.4.11 to 3.4.16. The plasma data for animals involved in this experiment are also shown in Appendix 1 (Table A1.3).

Figure 3.4.5 Autoradiographic images of the LCMRglu response to DOI



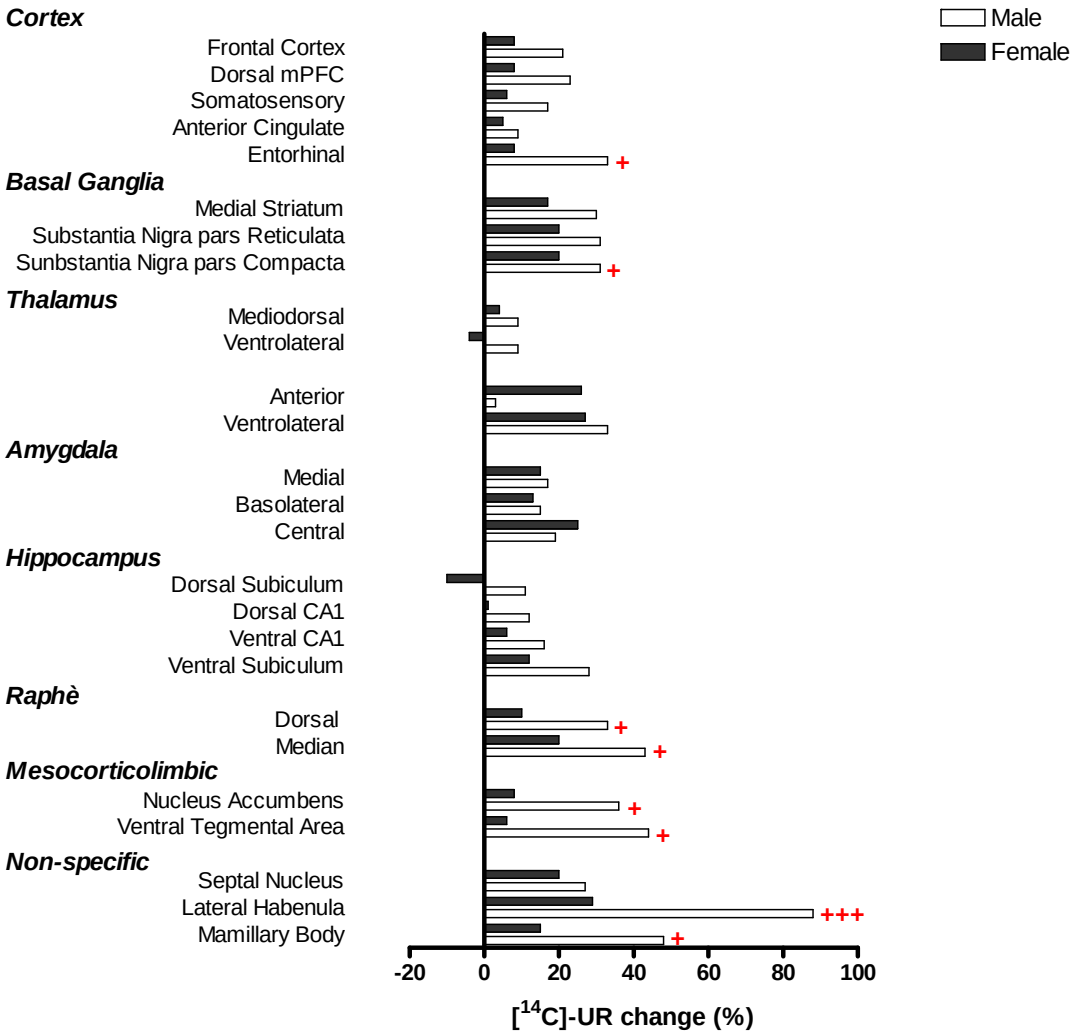
Representative "false-colour" autoradiographic images at the level of the caudate, dorsal hippocampus, ventral hippocampus and raphé in saline-treated and DOI-treated wild-type male mice. High levels of tissue isotope accumulation are shown as "warm" colours (red/orange) whilst low isotope tissue accumulation is depicted by "cold" colours (green/blue). Note in particular how DOI-treatment causes a wide-spread increase in isotope accumulation.

Figure 3.4.6 Effect of *h*SERT OVR on the LCMRglu response to DOI in representative brain regions



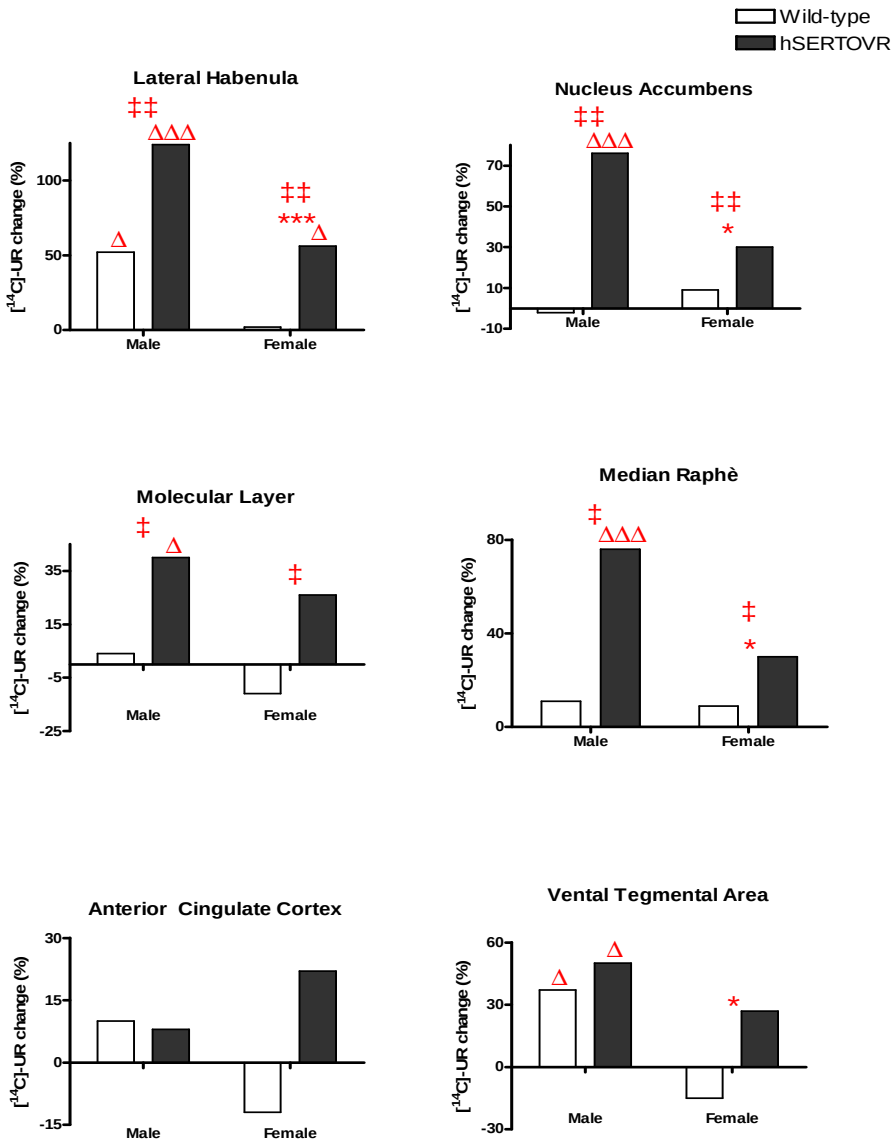
*LCMRglu response ([^{14}C]-Uptake Ratio) to 25mg.kg^{-1}DOI in 6 representative brain regions of Wt and hSERT OVR mice of both genders. Data were analysed using Univariate ANOVA with acceptable levels of significance set at p<0.05. *denotes p<0.05, ***denotes p<0.001 significant DOI response (Bonferroni post-hoc correction in comparison of means). +denotes p<0.05, +++denotes p<0.001 significant sex x DOI interaction. †denotes p<0.05, ††denotes p<0.01 significant hSERT OVR x DOI interaction. The LCMRglu response was significantly greater in males than in females in a number of brain regions (e.g. nucleus accumbens and median raphé). The response to DOI was significantly enhanced in hSERT OVR mice as compared to Wt mice in mice of both genders in a number of functionally diverse brain regions (e.g. lateral habenula and median raphé).*

Figure 3.4.7 Gender differences in the LCMRglu response to DOI



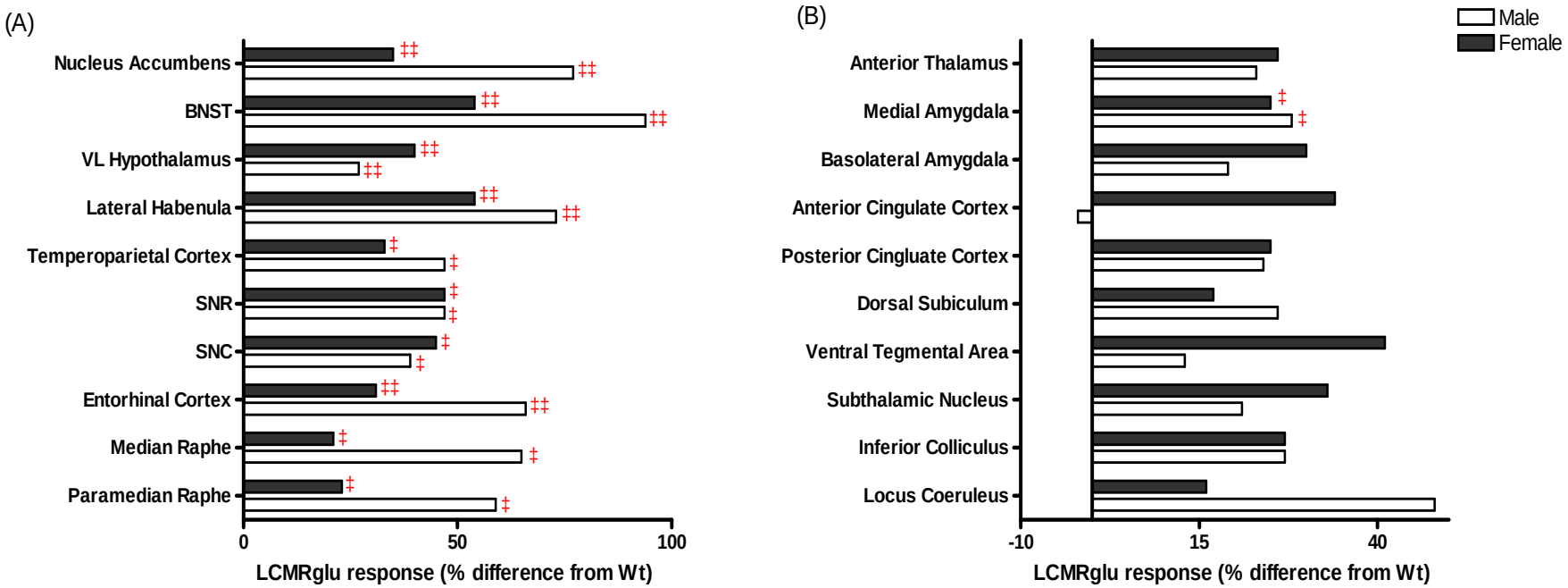
Influence of gender on the LCMRglu response to 25mg.kg^{-1} DOI. Data shown as % change in [^{14}C]-Uptake Ratio ([^{14}C]-UR) in-DOI treated animals relative to saline controls with data included from both Wt and hSERT OVR mice. $^{+}$denotes p<0.05 and $^{+++}$denotes p<0.001 significant gender x DOI interaction (Univariate ANOVA). The response to DOI is significantlydecreased in females as compared to males in a number of brain regions including the lateral habenula, mamillary body and raphé.

Figure 3.4.8 Effect of *h*SERT OVR on the LCMRglu response to DOI



*LCMRglu response to 25mg.kg^{-1}DOI in 6 representative brain regions of Wt and hSERT OVR mice of both genders. Data represented as % change in the [^{14}C]-Uptake Ratio ([^{14}C]-UR) in DOI-treated animals relative to the appropriate saline control. Data were analysed using Univariate ANOVA with acceptable levels of significance set at p<0.05. $^{\Delta}$denotes p<0.05, $^{\Delta\Delta}$denotes p<0.01, $^{\Delta\Delta\Delta}$denotes p<0.001 significant DOI response (Bonferroni post-hoc correction in comparison of means). *denotes p<0.05, ***denotes p<0.001 significant sex x DOI interaction. ‡denotes p<0.05, ‡‡denotes p<0.01 significant hSERT OVR x DOI interaction. The response to DOI was significantly increased by hSERT over-expression in mice of both genders in a number of brain regions including the lateral habenula, nucleus accumbens and median raphé.*

Figure 3.4.9 *h*SERT OVR effect on the LCMRglu response to DOI: maximum and minimum effect



Areas in which hSERT OVR has the greatest (A) and least (B) effect upon the LCMRglu response to DOI in male and female mice. Data presented as % difference in LCMRglu response to DOI ([^{14}C]-Uptake ratio) between hSERT OVR and wild-type animals. ‡denotes p<0.05, ‡‡denotes p<0.01 significant hSERT OVR x DOI interaction (Univariate ANOVA). The LCMRglu response to DOI was significantly increased by *hSERT over-expression in a number of brain regions. Despite the observation that the LCMRglu response to DOI may be enhanced to a greater extent by hSERT over-expression in males than in females in some brain regions (e.g. median raphé) this effect was not significant.*

Table 3.4.11 LCMRglu response to DOI in *h*SERT over-expressing mice: cortical regions

	Male						Female					
	Wild-type			hSERT OVR			Wild-type			hSERT OVR		
	Saline	DOI	%	Saline	DOI	%	Saline	DOI	%	Saline	DOI	%
Cortex												
Orbitofrontal	58 ± 6	62 ± 4	**6**	50 ± 5	72 ± 2	**49**†**	49 ± 4	45 ± 4	**-7**	31 ± 3	39 ± 4	**24†**
Frontal	43 ± 5	42 ± 4	**-2**	36 ± 4	52 ± 6	**44*†**	44 ± 3	36 ± 4	**-18**	25 ± 2	28 ± 3	**13†**
Anterior Cingulate	46 ± 5	50 ± 6	**10**	38 ± 4	41 ± 8	**8**	43 ± 3	38 ± 4	**-12**	27 ± 3	32 ± 3	**22**
Dorsal medial Prefrontal	44 ± 5	45 ± 5	**1**	35 ± 3	51 ± 6	**45***	37 ± 3	35 ± 4	**-6**	23 ± 2	28 ± 3	**23**
Ventral medial Prefrontal	40 ± 4	38 ± 4	**-3**	31 ± 3	44 ± 5	**40*†**	29 ± 2	27 ± 3	**-6**	17 ± 2	23 ± 2	**33†**
Somatosensory	51 ± 5	56 ± 6	**9**	46 ± 5	58 ± 4	**26**	55 ± 5	43 ± 4	**-14**	28 ± 3	35 ± 3	**27**
Temperoparietal	51 ± 5	52 ± 4	**1**	47 ± 5	69 ± 7	**48***	44 ± 4	39 ± 5	**-12+**	28 ± 2	34 ± 3	**21+**
Posterior Cingulate	49 ± 5	47 ± 4	**-5**	47 ± 5	55 ± 6	**19**	48 ± 4	39 ± 5	**-20**	32 ± 3	33 ± 3	**5**
Piriform	29 ± 3	36 ± 7	**25**	24 ± 2	34 ± 2	**43****	20 ± 2	21 ± 2	**5**	14 ± 2	18 ± 2	**32**
Entorhinal	34 ± 3	34 ± 3	**0**	30 ± 3	49 ± 3	**65***††**	30 ± 2	28 ± 3	**-8+**	19 ± 2	24 ± 2	**24+††**

Effect of *h*SERT over-expression on the LCMRglu ([^{14}C]-Uptake ratio) response to DOI in cortical regions. Data shown as mean ± s.e.m. and % difference between DOI-treated and control (saline-treated) animals of the same gender and genotype. *denotes p<0.05, **denotes p<0.01 and ***denotes p<0.001 significant DOI effect (within genotype 2-way ANOVA with Bonferroni correction). +denotes p<0.05 significant gender x DOI interaction. †denotes p<0.05, ††denotes p<0.01 significant genotype x DOI interaction (Univariate ANOVA).

Table 3.4.12 LCMRglu response to DOI in *h*SERT over-expressing mice: basal ganglia regions

Brain Region	Male						Female					
	Wild-type			*h*SERT OVR			Wild-type			*h*SERT OVR		
	Saline	DOI	%	Saline	DOI	%	Saline	DOI	%	Saline	DOI	%
Basal Ganglia												
Medial Striatum	41 ± 3	48 ± 5	**19**	38 ± 4	53 ± 4	**41***	42 ± 3	40 ± 4	**-3**	25 ± 3	34 ± 4	**37**
Lateral Striatum	46 ± 4	51 ± 5	**13**	40 ± 4	58 ± 6	**45***	42 ± 3	38 ± 4	**-9**	26 ± 3	38 ± 4	**26**
Globus Pallidus	30 ± 2	29 ± 6	**-6**	28 ± 3	38 ± 4	**18**†	33 ± 3	25 ± 3	**-22**	18 ± 2	23 ± 2	**27**†
Subthalamic Nucleus	46 ± 5	53 ± 5	**14**	45 ± 5	61 ± 5	**35***	45 ± 4	39 ± 4	**-14**	30 ± 3	36 ± 3	**19**
Substantia Nigra pars Reticulata	29 ± 2	31 ± 3	**7**	27 ± 2	41 ± 5	**54****†	26 ± 2	25 ± 3	**-4**	17 ± 2	24 ± 2	**44**†
Substantia Nigra pars Compacta	36 ± 3	42 ± 4	**15**	33 ± 3	51 ± 4	**54****†	34 ± 3	31 ± 3	**-9**$^{+}$	21 ± 2	29 ± 3	**36**$^{+\dagger}$

Effect of *h*SERT over-expression on the LCMRglu response to DOI in Basal Ganglia regions. Data shown as mean ± s.e.m. and % difference between DOI-treated and control (saline-treated) animals of the same gender and genotype. *denotes p<0.05, **denotes p<0.01 and ***denotes p<0.001 significant DOI effect (within gender and genotype, 2-way ANOVA with Bonferroni correction). $^{+}$denotes p<0.05 significant gender x DOI interaction. †denotes p<0.05 significant genotype x DOI interaction (Univariate ANOVA).

Table 3.4.13 LCMRglu response to DOI in hSERT over-expressing mice: amygdala, thalamic and hypothalamic regions

	Male						Female					
	Wild-type			*h*SERT OVR			Wild-type			*h*SERT OVR		
	Saline	**DOI**	**%**	**Saline**	**DOI**	**%**	**Saline**	**DOI**	**%**	**Saline**	**DOI**	**%**
Amygdala												
Medial	24 ± 2	25 ± 2	**3**	21 ± 2	27 ± 2	**31**	18 ± 2	18 ± 2	**3**	12 ± 2	15 ± 1	**28**
Basolateral	33 ± 2	34 ± 3	**5**	30 ± 3	37 ± 3	**24**	29 ± 2	28 ± 2	**-2**	18 ± 2	23 ± 2	**28**
Central	22 ± 2	23 ± 2	**7**	19 ± 2	25 ± 2	**31**	17 ± 2	19 ± 2	**15**	12 ± 2	16 ± 1	**36**
Thalamic Nuclei												
Anterior	47 ± 4	46 ± 4	**-3**	47 ± 5	57 ± 5	**21**	47 ± 5	40 ± 4	**-16**	32 ± 3	35 ± 4	**10**
Mediodorsal	54 ± 5	51 ± 5	**-7**	47 ± 4	59 ± 6	**25**†	48 ± 4	42 ± 4	**-13**	29 ± 3	35 ± 3	**21**†
Venterolateral	53 ± 5	50 ± 5	**-5**	50 ± 5	61 ± 8	**22**†	56 ± 5	47 ± 6	**-16**	31 ± 3	36 ± 3	**16**†
Hypothalamic Nuclei												
Anterior	37 ± 6	33 ± 4	**-12**	34 ± 4	40 ± 6	**18**	19 ± 3	20 ± 2	**3**	13 ± 2	19 ± 2	**50**
Venterolateral	28 ± 2	33 ± 3	**16**	27 ± 3	41 ± 3	**50****	26 ± 2	27 ± 3	**3**$^{+}$	16 ± 2	24 ± 2	**50**$^{+}$

Effect of *h*SERT over-expression on the LCMRglu response to DOI in amygdala, thalamic and hypothalamic regions. Data shown as mean ± s.e.m. and % difference between DOI-treated and control (saline-treated) animals of the same gender and genotype. *denotes p<0.05, **denotes p<0.01 and ***denotes p<0.001 significant DOI effect (within gender and genotype, 2-way ANOVA with Bonferroni correction). †denotes p<0.05, ††denotes p<0.01 significant genotype x DOI interaction (Univariate ANOVA).

Table 3.4.14 LCMRglu response to DOI in *h*SERT over-expressing mice: hippocampal regions

	Male						Female					
	Wild-type			hSERT OVR			Wild-type			hSERT OVR		
	Saline	DOI	%	Saline	DOI	%	Saline	DOI	%	Saline	DOI	%
Hippocampus												
Molecular Layer	41 ± 4	42 ± 4	**4**	34 ± 3	48 ± 5	**40*†**	32 ± 3	31 ± 5	**-5**	21 ± 2	25 ± 3	**20†**
Dorsal Subiculum	38 ± 3	37 ± 3	**-2**	34 ± 3	42 ± 3	**24**	38 ± 3	33 ± 5	**-13**	25 ± 3	24 ± 2	**-5**
Dentate PO	25 ± 1	24 ± 2	**-3**	22 ± 2	27 ± 2	**24**	20 ± 2	20 ± 3	**-2**	15 ± 2	16 ± 2	**8**
Dorsal CA1	35 ± 3	33 ± 3	**-5**	30 ± 2	38 ± 2	**30*†**	28 ± 3	25 ± 4	**-10**	17 ± 2	20 ± 2	**13†**
CA2	33 ± 3	31 ± 3	**-7**	27 ± 3	36 ± 3	**33*†**	26 ± 3	23 ± 12	**-12**	16 ± 2	19 ± 2	**20†**
Ventral CA1	33 ± 2	31 ± 3	**-6**	28 ± 3	38 ± 3	**38*†**	24 ± 2	23 ± 10	**-4**	17 ± 2	19 ± 2	**14†**
Ventral Subiculum	29 ± 2	33 ± 3	**13**	26 ± 3	37 ± 3	**43****	23 ± 2	24 ± 4	**-3**	17 ± 2	21 ± 2	**21**
CA3	27 ± 2	26 ± 2	**4**	24 ± 2	32 ± 3	**34**	21 ± 2	19 ± 3	**-8**	14 ± 2	16 ± 2	**20**

Effect of *h*SERT over-expression on the LCMRglu response to DOI in hippocampal regions. Data shown as mean ± s.e.m. and % difference between DOI-treated and control (saline-treated) animals of the same gender and genotype. *denotes p<0.05, **denotes p<0.01 and ***denotes p<0.001 significant DOI effect (within gender and genotype, 2-way ANOVA with Bonferroni correction). $^{+}$denotes p<0.05 significant gender x DOI interaction. †denotes p<0.05 significant genotype x DOI interaction (Univariate ANOVA).

Table 3.4.15 LCMRglu response to DOI in hSERT over-expressing mice: raphé and mesocorticolimbic regions

	Male						Female					
	Wild-type			hSERT OVR			Wild-type			hSERT OVR		
	Saline	DOI	%	Saline	DOI	%	Saline	DOI	%	Saline	DOI	%
Raphé												
Dorsal	36 ± 3	45 ± 5	**23**	31 ± 3	45 ± 4	**44***	29 ± 2	28 ± 3	**-4**$^{+}$	18 ± 2	22 ± 2	**20**$^{+}$
Median	46 ± 5	51 ± 5	**11**	39 ± 4	69 ± 6	**76*****†	39 ± 3	42 ± 6	**9**$^{+}$	25 ± 3	30 ± 4	**23**$^{+\dagger}$
Paramedian	45 ± 4	52 ± 4	**17**	39 ± 4	68 ± 5	**76*****†	40 ± 3	42 ± 6	**6**$^{+}$	25 ± 3	31 ± 3	**23**$^{+\dagger}$
Mesocorticolimbic System												
Ventral Tegmental Area	36 ± 3	50 ± 6	**37***	37 ± 5	56 ± 6	**50***	43 ± 4	38 ± 6	**-11**$^{+}$	28 ± 3	33 ± 4	**22**$^{+}$
Nucleus Accumbens	43 ± 6	42 ± 4	**-2**	34 ± 3	59 ± 3	**74*****††	31 ± 2	30 ± 5	**-4**$^{+}$	19 ± 2	23 ± 2	**20**$^{+\dagger\dagger}$

Effect of *h*SERT over-expression on the LCMRglu response to DOI raphé, mesocorticolimbic and non-specific brain regions. Data shown as mean ± s.e.m. and % difference between DOI-treated and control (saline-treated) animals of the same gender and genotype. *denotes p<0.05, **denotes p<0.01 and ***denotes p<0.001 significant DOI effect (within genotype, 2-way ANOVA with Bonferroni correction). $^{+}$denotes p<0.05, $^{++}$denotes p<0.05 and $^{+++}$denotes p<0.001 significant gender x DOI interaction. †denotes p<0.05, ††denotes p<0.01 significant genotype x DOI interaction (Univariate ANOVA).

Table 3.4.16 LCMRglu response to DOI in hSERT over-expressing mice: non-specific regions

	Male						Female					
	Wild-type			hSERT OVR			Wild-type			hSERT OVR		
	Saline	DOI	%	Saline	DOI	%	Saline	DOI	%	Saline	DOI	%
Non-specific												
Septal Nucleus	34 ± 3	35 ± 3	**3**	29 ± 3	44 ± 5	**51*†**	29 ± 2	31 ± 5	**6**	18 ± 2	25 ± 3	**34***†**
Bed Nucleus of the Stria Terminalis	27 ± 3	24 ± 3	**-12**	21 ± 2	39 ± 4	**82***††**	17 ± 2	20 ± 3	**19**	11 ± 2	18 ± 2	**61*††**
Corpus Callosum	16 ± 1	14 ± 1	**-12**	13 ± 1	20 ± 2	**50***	11 ± 2	15 ± 2	**37**	8 ± 1	12 ± 1	**54**
Lateral Habenula	53 ± 5	81 ± 10	**52***	48 ± 5	107 ± 10	**124***††**	55 ± 4	57 ± 9	**5^{+++}**	32 ± 3	48 ± 5	**50*$^{+++\dagger\dagger}$**
Mamillary Body	52 ± 4	72 ± 8	**40***	50 ± 5	78 ± 5	**56****	56 ± 3	53 ± 7	**-5^{+}**	34 ± 3	43 ± 5	**27^{+}**
Periaqueductal Grey	33 ± 3	35 ± 1	**7**	26 ± 2	37 ± 4	**40***	23 ± 3	23 ± 3	**0**	15 ± 2	19 ± 2	**25**
Inferior Colliculus	73 ± 7	89 ± 9	**22**	71 ± 10	105 ± 12	**50***	81 ± 7	77 ± 6	**-5**	48 ± 5	56 ± 7	**16**
Ventral Tegmental Nucleus	46 ± 4	58 ± 6	**26**	41 ± 4	67 ± 5	**65*****	43 ± 3	46 ± 6	**5**	26 ± 2	32 ± 2	**14**
Locus Coeruleus	41 ± 4	51 ± 6	**24**	36 ± 4	62 ± 5	**71*****	38 ± 3	38 ± 4	**2**	22 ± 2	25 ± 2	**14**
Nucleus Tractus Solitaris	56 ± 9	58 ± 12	**4**	44 ± 7	75 ± 13	**69**	37 ± 4	42 ± 6	**17**	17 ± 1	24 ± 3	**40**

Effect of *h*SERT over-expression on the LCMRglu response to DOI raphé, mesocorticolimbic and non-specific brain regions. Data shown as mean ± s.e.m. and % difference between DOI-treated and control (saline-treated) animals of the same gender and genotype. *denotes p<0.05, **denotes p<0.01 and ***denotes p<0.001 significant DOI effect (within genotype, 2-way ANOVA with Bonferroni correction). $^{+}$denotes p<0.05, $^{++}$denotes p<0.05 and $^{+++}$denotes p<0.001 significant gender x DOI interaction. †denotes p<0.05, ††denotes p<0.01 significant genotype x DOI interaction (Univariate ANOVA).

4.5 Conclusions and Discussion

In this study we found evidence for a widespread reduction in 5-HT_{2A} receptor binding and a reduction in 5-HT_{2C} receptor binding that was limited to the nucleus accumbens of females as compared to males. Due to the nature of the binding protocol used in this study, which utilised a non-saturating concentration of each radioligand, we are unable to determine if this alteration in binding reflects a decrease in the expression level or affinity of these receptors. Further research is required to clarify this issue. The gender difference in 5-HT_{2A} binding reported in this study parallels that reported in humans (Biver et al., 1996). However, other studies have reported no gender difference in humans (Adams et al., 2004) while significantly increased 5-HT_{2A} binding in the hippocampus of female rats as compared to males (Zhang et al., 1999) has also been reported. The reason for disparities in these results is unclear, but may relate to the use of small sample sizes in those studies reporting no gender difference, species differences or the use of different radioligands in each study. To the best of our knowledge our study is the first to characterise gender differences in 5-HT_{2C} receptor binding. It is of particular interest that we found 5-HT_{2C} binding was significantly reduced in the nucleus accumbens but significantly increased in the choroid plexus of females as compared to males. This suggests that the gender-dependent regulation of 5-HT_{2C} receptor pharmacology involves diverse mechanisms that differ in neurones, particularly those of the nucleus accumbens, and the epithelial cells of the choroid plexus.

We found that 25mg.kg^{-1} DOI produced only significant increases in cerebral metabolism. These findings contrast to those previously reported by the quantitative 2-DG method in rats, where only significant decreases were found in response to this dose of DOI (Freo et al., 1992; Freo et al., 1991). The reason for this disparity is unknown. However, the localisation of the increased metabolism noted in mice (Wt male: lateral habenula, ventral tegmental area, mamillary body) in response to DOI differs from those in which significant decreases were detected in rats. Indeed, in no brain region showing decreased metabolism in response to 25mg.kg^{-1} DOI in rats was a response noted in mice with this dose. Furthermore, we found that a lower dose of DOI (2.5mg.kg^{-1}) in male Wt mice (unpublished observations) produced significant decreases in cerebral metabolism that paralleled those reported in rats (at both 2.5mg.kg^{-1} and 25mg.kg^{-1}) (Freo et al., 1991). Therefore, it may be suggested that the sensitivity of the 5-$HT_{2A/C}$ receptors responsible for this divergent response to DOI may be different between species and may contribute to the divergent metabolic response to 25mg.kg^{-1} DOI between rats and mice.

In parallel to our binding data showing that 5-$HT_{2A/C}$ receptor binding is lower in females than in males we also found that the cerebral metabolic response to the 5-$HT_{2A/C}$ agonist DOI was lower in females than in males. The widespread decrease in 5-HT_{2A} binding reported in females is likely to represent a primary mechanism by which 5-$HT_{2A/C}$ functional responses are decreased in females as compared to males. However, some caution should be exercised when drawing this conclusion as the receptor binding characterised in this study involves the use of receptor antagonists that label total receptor levels rather than those in the high affinity state coupled to G-proteins. Therefore, it may be possible that total 5-$HT_{2A/C}$ receptor expression is different between the genders but the level of those receptors in the high affinity state, coupled to G-proteins, may not be different between the genders. This seems unlikely, however, given the magnitude of the gender differences reported in 5-HT_{2A} binding in some brain regions. However, additional mechanisms including altered coupling efficiency of the 5-$HT_{2A/C}$ receptors between genders can not be ruled out. Our data not only suggest that 5-$HT_{2A/C}$ receptor functioning is decreased in females but also that basal levels of 5-$HT_{2A/C}$ receptor activation is likely to be lower in females than in males. This seems even more likely given that we have found evidence for increased SERT expression in females (study 2), suggestive of enhanced 5-HT reuptake, and that others have reported decreased extracellular 5-HT levels in females as compared to males (Jones and Lucki, 2005; Mitsushima et al., 2006).

We found that 5-HT_{2A} binding was not altered in any brain regions in *h*SERT OVR mice. This is in contrast to the reported alterations in 5-HT_{2A} receptor binding in animals where SERT function is completely ablated, where both increases and decreases have been reported (Li et al., 2003; Rioux et al., 1999). Although the reason for this disparity between these two animal models is unknown the differing magnitudes of alteration in extracellular 5-HT levels is likely to be a contributory mechanism. In SERT KO animals extracellular 5-HT is increased by approximately +420% to +1285% (Shen et al., 2004) whereas in *h*SERT OVR animals it is decreased by a more modest -55% (Jennings et al., 2006). In addition, the observation that SERT binding is altered in a heterogeneous, brain-regions dependent manner in *h*SERT OVR mice (study 2) suggests that this mouse model does not represent a direct diametric reflection of the SERT KO mouse, where SERT expression is completely removed in all brain regions. Therefore, complex differences in the regulation of 5-HT system functioning in response to the genetically altered SERT expression present in these animal models is likely to occur. Our results suggest that alterations in 5-HT_{2C} receptor pharmacology may be more sensitive to alterations in SERT functioning than the 5-HT_{2A} receptor as we found evidence for localised alterations in [^{3}H]mesulergine binding in *h*SERT OVR mice. 5-HT_{2C} receptor binding was significantly increased in the Dorsal mPFC and

choroid plexus in *h*SERT OVR animals. Surprisingly, an increase in 5-HT_{2C} binding has also been reported in the choroid plexus of SERT KO mice (Li et al., 2003). This suggests that alterations in 5-HT_{2C} receptor binding in this region may not directly relate to alterations in extracellular 5-HT levels and the relevance of this alteration in receptor function to affective behaviour, if any, remains unclear. Furthermore, our study also supports the contention that 5-HT_{2C} receptor pharmacology is altered by genetically determined SERT expression in a brain region-dependent manner, as a decrease in 5-HT_{2C} binding found in the ventrolateral hypothalamus.

Despite the limited effect of *h*SERT OVR on 5-HT_{2A} and 5-HT_{2C} receptor binding we found that 5-$HT_{2A/C}$ mediated functional responses were significantly increased in the majority of brain regions in these animals. As the level of cerebral metabolism following DOI treatment in *h*SERT OVR mice often surpasses that following DOI treatment in Wt mice it is unlikely that the enhanced response to DOI in *h*SERT OVR mice can be attributed to the constitutive hypo-metabolism in these animals and that the response in Wt animals is limited by their closer basal proximity to the maximum DOI response ("ceiling effect"). Our data, therefore, support the contention that 5-$HT_{2A/C}$ functioning is enhanced in *h*SERT OVR mice. This is consistent with the reported enhancement of DOI induced c-fos expression in *h*SERT OVR as compared to Wt animals (Jennings et al., 2003). Furthermore, this finding is not only consistent with an adaptive functional response in these receptors to the decreased extracellular 5-HT levels in *h*SERT OVR mice but also parallels the findings of 5-$HT_{2A/C}$ function in SERT KO animals (Qu et al., 2005). Our results suggest that alterations in 5-HT_{2C} receptor pharmacology (expression level or affinity) may contribute to some of the alterations observed in 5-$HT_{2A/C}$ functional responses. However, as alterations in 5-HT_{2C} receptor pharmacology are limited in their distribution and 5-HT_{2A} receptor pharmacology unaltered, in contrast to the widespread enhancement of 5-$HT_{2A/C}$ receptor function other mechanisms are likely to be important. This may include the enhanced coupling of these receptors to their effector G-proteins and down-stream coupling mechanisms. With regard to possible mechanisms involving the 5-HT_{2C} receptor, alterations in mRNA editing resulting in the presence of different 5-HT_{2C} receptor isoforms that possess different coupling efficiencies to G-proteins (Niswender et al., 1999) may contribute to the enhanced 5-$HT_{2A/C}$ functional response in *h*SERT OVR mice. Indeed, there is evidence that prolonged 5-HT depletion results in the expression of 5-HT_{2C} receptor isoforms with enhanced coupling (Gurevich et al., 2002a) suggesting that the decreased extracellular 5-HT levels in *h*SERT OVR mice may result in this. Furthermore, there is some evidence that alternative 5-HT_{2C} receptor mRNA editing can alter the binding affinity of these receptors (Fitzgerald et al., 1999). Therefore, 5-HT_{2C} RNA editing in the prefrontal cortex may contribute to the altered 5-HT_{2C} receptor

binding in this brain regions in *h*SERT OVR mice. These data suggest that the possibility of altered 5-HT_{2C} mRNA editing *h*SERT OVR mice should be explored in the future. Furthermore, altered 5-HT_{2C} RNA editing (Gurevich et al., 2002b) consistent with reduced coupling efficiency has been noted in the prefrontal cortex of depressed suicide victims. Investigating the possible alteration of 5-HT_{2C} mRNA editing in the prefrontal cortex of *h*SERT OVR mice may be relevant to both the noted alterations in 5-HT_{2C} binding in this region and the affective functioning of these animals. An alternative mechanism that may allow for enhanced signal transduction following receptor activation may involve increased levels of the G-proteins which couple to these receptors. This would increase the level of the high affinity forms of these receptors. However, evidence from SERT KO mice (Li et al., 2003) and chronic SSRI studies (Li et al., 1997a) suggest that the altered coupling efficiency of 5-$HT_{2A/C}$ receptors following chronic alterations in 5-HT transmission is independent of alterations in the expression level of 5-$HT_{2A/C}$ receptor linked G-proteins.

In these studies we found no evidence to suggest that 5-$HT_{2A/C}$ receptor binding or functioning was altered to a greater extent by *h*SERT OVR in females as compared to males. Therefore, it is unlikely that the altered functioning of these receptors is involved in the gender-dependent effects of *h*SERT OVR on the constitutive cerebral hypometabolism, where the effect is greater in females than in males. Enhanced 5-$HT_{2A/C}$ receptor function in *h*SERT OVR mice may represent a compensatory mechanism to maintain a similar level of 5-$HT_{2A/C}$ receptor signalling in these animals in the presence of reduced extracellular 5-HT levels. However, it is still unclear as to whether the enhanced 5-$HT_{2A/C}$ receptor function in these animals is sufficient to maintain a normal level of 5-$HT_{2A/C}$ receptor activation. Indeed, the finding that 5-$HT_{2A/C}$ mediated functional responses are not significantly enhanced in some brain regions argues that the receptors mediating them would be hypo-stimulated in *h*SERT OVR mice under basal conditions. Furthermore, as evidence from both 5-HT_{2A} and 5-HT_{2C} receptor KO mice suggests that the activation of these receptors is anxiogenic as both of these transgenic lines display decreased anxiety-like behaviour (Weisstraub et al., 2006, Heisler et al., 1999; Heisler et al., 2007; Tecott et al., 1996) hypostimulation of these receptors in SERT OVR mice wqould be consistent with their anxiolytic behavioural phenotype. Clearly, further research should be dedicated to elucidating 5-$HT_{2A/C}$ receptor function under basal condition in *h*SERT OVR mice. Using of selective 5-HT_{2A} and 5-HT_{2C} receptor antagonists would not only allow for the assessment of basal 5-$HT_{2A/C}$ receptor function in *h*SERT OVR mice but would also allow for further characterisation of the individual receptor subtypes in these animals.

In summary, we found evidence for sexual dimorphism in both 5-$HT_{2A/C}$ receptor pharmacology and function, with females showing lower levels of function than those observed in males. In addition we found that a life-long increase in SERT expression altered 5-HT_{2C} receptor pharmacology, but not 5-HT_{2A} receptor pharmacology, in a brain-region dependent manner and resulted in increased 5-$HT_{2A/C}$ receptor function. While these functional differences in 5-$HT_{2A/C}$ may relate to the reduced anxiety in *h*SERT OVR mice it is unlikely that they contribute to the gender-dependent modulation of *h*SERT OVR on cerebral functioning, and so the possible interaction between gender and a life-long increase in SERT expression on affective functioning.

Study 5- 5-HT_{1B} receptor binding and function in *h*SERT over-expressing mice

5.1 Rationale

5-HT_{1B} receptors function as both terminal autoreceptors, inhibiting the release and synthesis of 5-HT (Hjorth and Tao, 1991), and heteroreceptors regulating the release of neurotransmitters from non-serotonergic neurones (Sari, 2004). Therefore, 5-HT_{1B} receptors have a central role in the regulation of 5-HT neurotransmission and in the integration between the 5-HT and other neurotransmitter systems. Evidence from chronic SSRI treatment studies support the contention that 5-HT_{1B} autoreceptor function down-regulates in response to enhanced synaptic 5-HT levels (Moret and Briley, 1990; Newman et al., 2004; Shalom et al., 2004). An effect which appears to be independent of alterations in 5-HT_{1B} receptor expression levels (Le Poul et al., 2000). However, other have reported that 5-HT_{1B} autoreceptor function is not altered by chronic SSRI treatment (Bosker et al., 1995; Gobbi et al., 1997). The contradictory evidence for 5-HT_{1B} regulation by extracellular 5-HT appears to by due, in part, to the central localisation in which 5-HT_{1B} receptor function is assessed. Hippocampal and hypothalamic 5-HT_{1B} autoreceptors do not appear to be regulated by extracellular 5-HT whereas those in the frontal cortex are. No study to date has specifically assessed the possible regulation of 5-HT_{1B} heteroreceptor function by chronic SSRI treatment or alterations in extracellular 5-HT. Evidence from studies using SERT KO mice suggests that 5-HT_{1B} receptor function can also be regulated by genetically determined SERT expression levels (Fabre et al., 2000b). In these animals 5-HT_{1B} function (as assessed by GTPγS binding) was found to be reduced in the substantia nigra. Furthermore, this alteration in 5-HT_{1B} function was attributed, in part, to a reduction in 5-HT_{1B} binding in this region. Given the available evidence we hypothesis that 5-HT_{1B} function may be up-regulated in *h*SERT OVR mice in response to the decreased synaptic 5-HT levels in these animals. Furthermore, we hypothesise that 5-HT_{1B} receptor binding may also be increased in *h*SERT OVR mice. Assessing 5-HT_{1B} receptor function in *h*SERT OVR mice by use of the [^{14}C]-2-DG imaging technique may allow us to further determine the CNS localisation at which 5-HT_{1B} receptor function may be regulated by SERT expression and alterations in extracellular 5-HT. Use of the 2-DG technique may be particularly appropriate in assessing the localisation of functional alterations in 5-HT_{1B} receptor given the synaptic localisation of these receptors, their inhibitory effect on neurotransmitter release and the sensitivity of the 2-DG method to alterations in metabolic demand at the nerve terminal.

The paucity of data regarding possible gender differences in 5-HT_{1B} receptor binding and function restricts our ability to form a decisive hypothesis on this matter. However, as the behavioural and neurochemical alterations observed in 5-HT_{1B} receptor KO mice appear to be greater in females than in males (Jones and Lucki, 2005) this at least suggests that 5-HT_{1B} function may be greater in females than in males. Furthermore, enhanced 5-HT_{1B} receptor function in females would be consistent with an adaptive response to the reduced extracellular 5-HT levels observed in females as compared to males (Jones and Lucki, 2005; Mitsushima et al., 2006). In order to assess possible gender differences in 5-HT_{1B} receptor function and the possibility of an interaction between gender, genetically determined SERT function and the 5-HT_{1B} receptor we will characterise 5-HT_{1B} receptor binding and function in Wt and *h*SERT OVR animals of both genders.

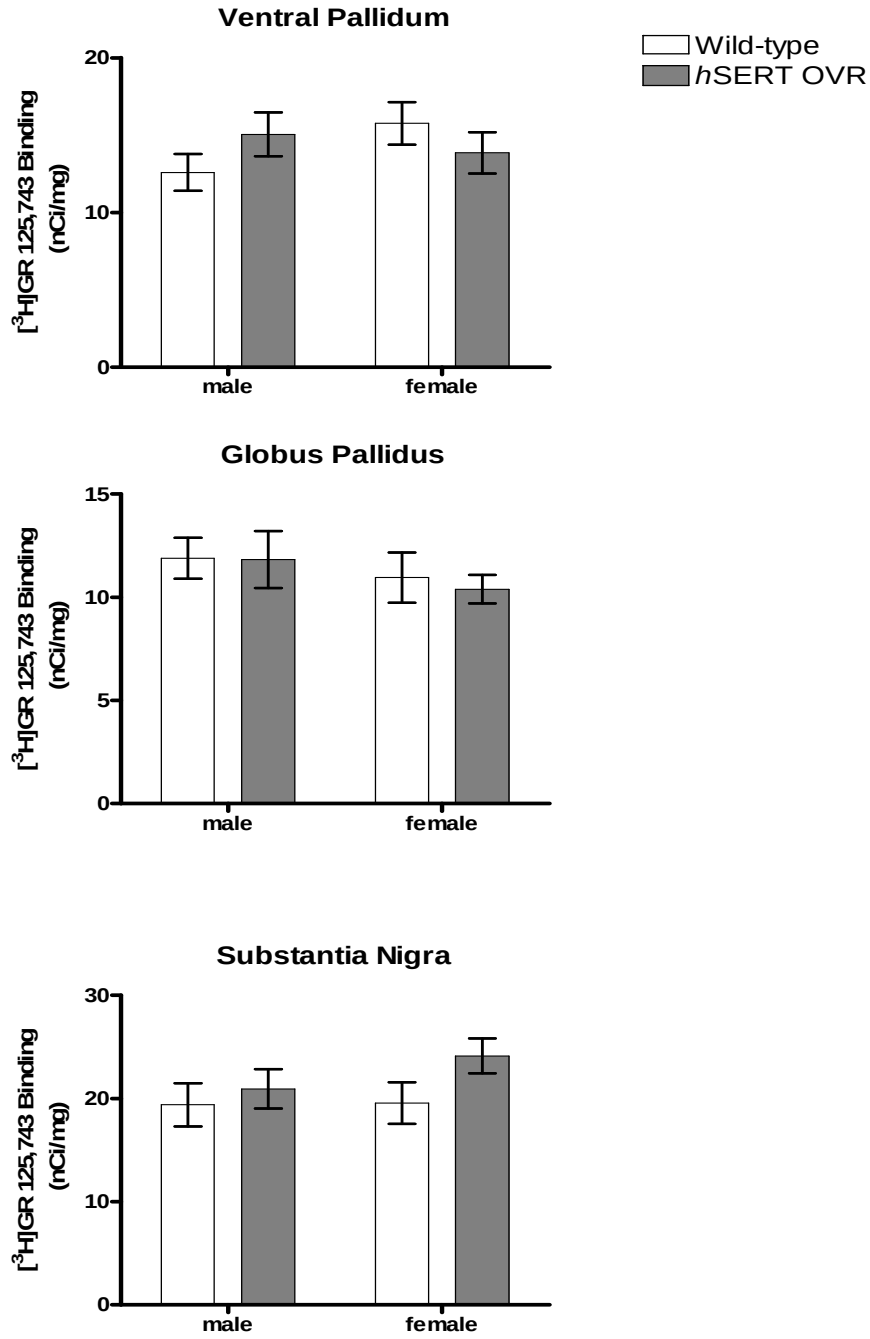
In summary, we hypothesis that 5-HT_{1B} receptor binding and function may be increased in *h*SERT OVR mice in comparison to Wt animals in an attempt to compensate for the reduced synaptic 5-HT levels in these animals. We also hypotheses that 5-HT_{1B} receptor function may be greater in females than in males and that any effect of *h*SERT OVR on 5-HT_{1B} function may also be greater in females than males, as our previous studies have shown this gender difference in constitutive cerebral metabolism, alterations in SERT expression levels and in 5-HT_{1A} receptor function.

5.2 [^{3}H]GR 125,743 Binding

In contrast to the widespread distribution of 5-HT_{1B} receptor binding reported in other studies (Fabre et al., 2000b; Le Poul et al., 2000; Mendelson and McEwen, 1992) we found that [^{3}H]GR 125,743 binding could only be visualised in 3 brain regions; the ventral pallidum, globus pallidus and substantia nigra. These regions are reported to display the highest 5-HT_{1B} binding when using other 5-HT_{1B} ligands suggesting that the protocol used for [^{3}H]GR 125,743 binding in this study requires further optimisation to measure 5-HT_{1B} binding in regions where expression of the receptor is more modest. In particular we note that the wash time utilised in this protocol is much longer than that utilised for ligands with a similar affinity for other receptors. Therefore, reducing the wash time may allow for determination of 5-HT_{1B} binding in regions in which expression is moderate or low.

There was no evidence for a significant gender difference in the extent of [^{3}H]GR 125,743 binding in any of the regions analysed. Furthermore, there was no evidence for a significant effect of *h*SERT over-expression on [^{3}H]GR 125,743 in any of these brain regions in either males or females (Figure 3.5.1).

Figure 3.5.1 Effect of *h*SERT on [^{3}H]GR 125,743 binding



[^{3}H]GR 125,743 binding (nCi/mg) in male and female hSERT OVR and wild-type mice. Data presented as mean ± s.e.m. There was no evidence for a difference in [^{3}H]GR 125,743 binding between the different genders or genotypes.

5.3 LCMRglu

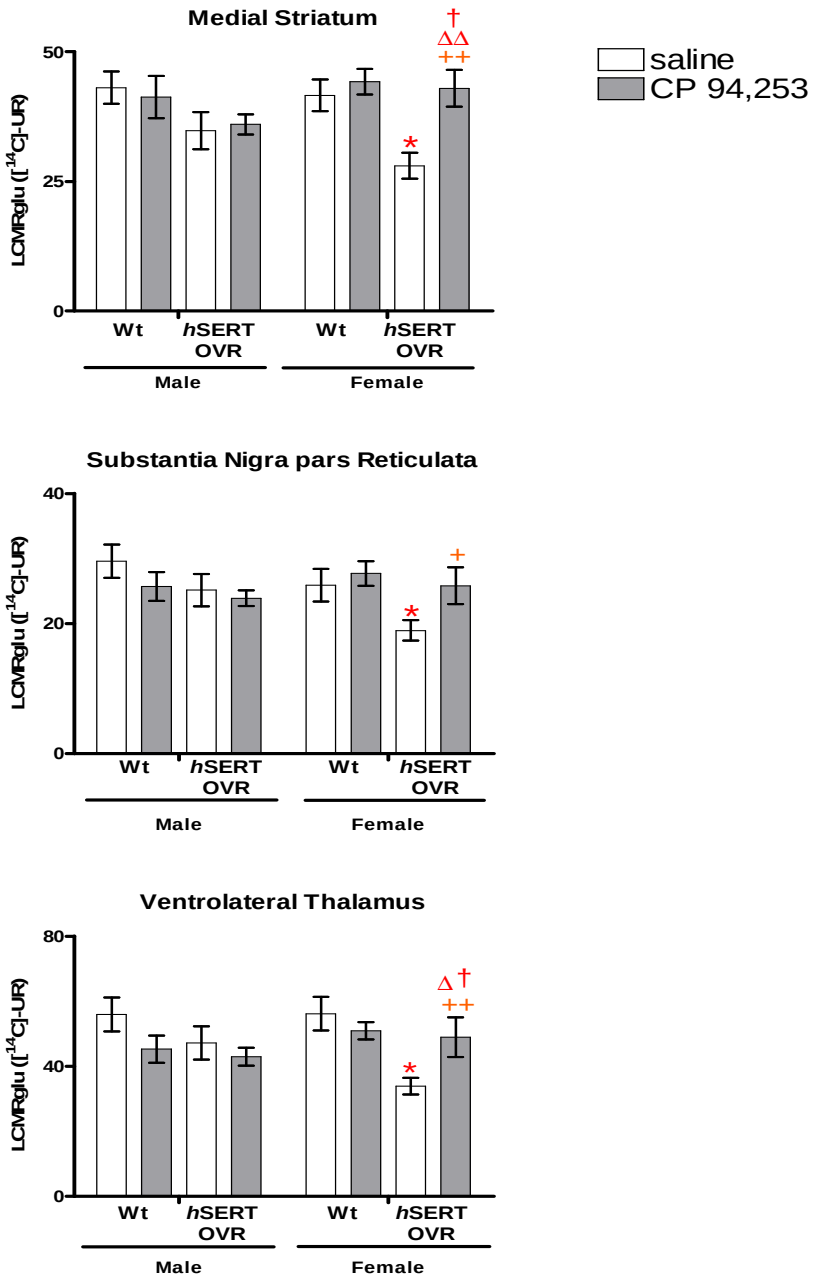
In Wt males CP 94,253 produced a general trend towards decreased LCMRglu in the majority of brain regions. However, the only region in which a significant decrease was observed in the anterior hypothalamus (-33%). Non-significant increases in LCMRglu in response to CP 94,253 were observed in 4 brain regions. In contrast to Wt males CP 94,253 increased LCMRglu in Wt females in a greater number of brain regions (35 out of 47). These increases were significant in a number of hippocampal subregions including the dentate PO (+22%), ventral subiculum (+25%), ventral CA1 (+27%) and CA3 (+25%). Out-with the hippocampus significant increases were observed only in the anterior hypothalamus (+41%) and central amygdala (+32%) of Wt females. A trend towards decreased LCMRglu was observed in 12 brain regions in Wt females but none reached significance. These results suggested that the LCMRglu response to CP 94,253 was greater in females than in males and this was confirmed by the observation of a significant gender x CP 94,253 interaction in 8 of the 46 brain regions analysed (2-way ANOVA within genotype). These significant interactions were most widespread throughout the hippocampus and were also present in the piriform cortex and anterior hypothalamus. Figures 3.5.2 and 3.5.3 show gender differences in the LCMRglu response to CP 94,253 in a representative selection of brain regions.

In *h*SERT OVR males the LCMRglu response to CP 94,253 was of a similar magnitude and distribution to that observed in Wt males and the only region in which a significant response was found was a significant reduction (-25%) in LCMRglu following CP 94,253 treatment in the anterior hypothalamus. There was no evidence for a significant interaction between *h*SERT OVR and CP 94,253 on LCMRglu any brain region in male mice. In contrast to the CP 94,253 response in *h*SERT OVR males CP 94,253 produced a tendency towards increased LCMRglu in all brain regions analysed in *h*SERT OVR females. Significant increases in LCMRglu in response to CP 94,253 were more widespread (20 regions) in female *h*SERT OVR than female Wt mice. Additional significant increases in LCMRglu were observed in cortical (orbitofrontal, +34%; somatosensory +38%), mesocorticolimbic (nucleus accumbens, +45%; VTA, 54%), motor (medial striatum +53%) and raphè (dorsal, +44%) structures in *h*SERT OVR female mice in comparison to Wt females. Furthermore, in all brain regions analysed the LCMRglu response to CP 94,253 tended to be of a greater magnitude in *h*SERT OVR females in comparison to their Wt counterparts. Indeed, a significant *h*SERT OVR x CP 94,253 interaction was found in females in 6 brain regions (frontal cortex, medial striatum, subthalamic nucleus, ventrolateral thalamus, mediodorsal thalamus and lateral habenula). These results suggested that *h*SERT OVR increases the LCMRglu response to CP 94,256 in females but not in males. Figure 3.5.4 shows a selection of representative brain regions in

which the LCMRglu response to CP 94,253 appears to be modified by *h*SERT over-expression to a greater extent in females as compared to males. However, there was no evidence for a significant gender x *h*SERT OVR x CP 94,253 interaction in any brain region which would support this conclusion.

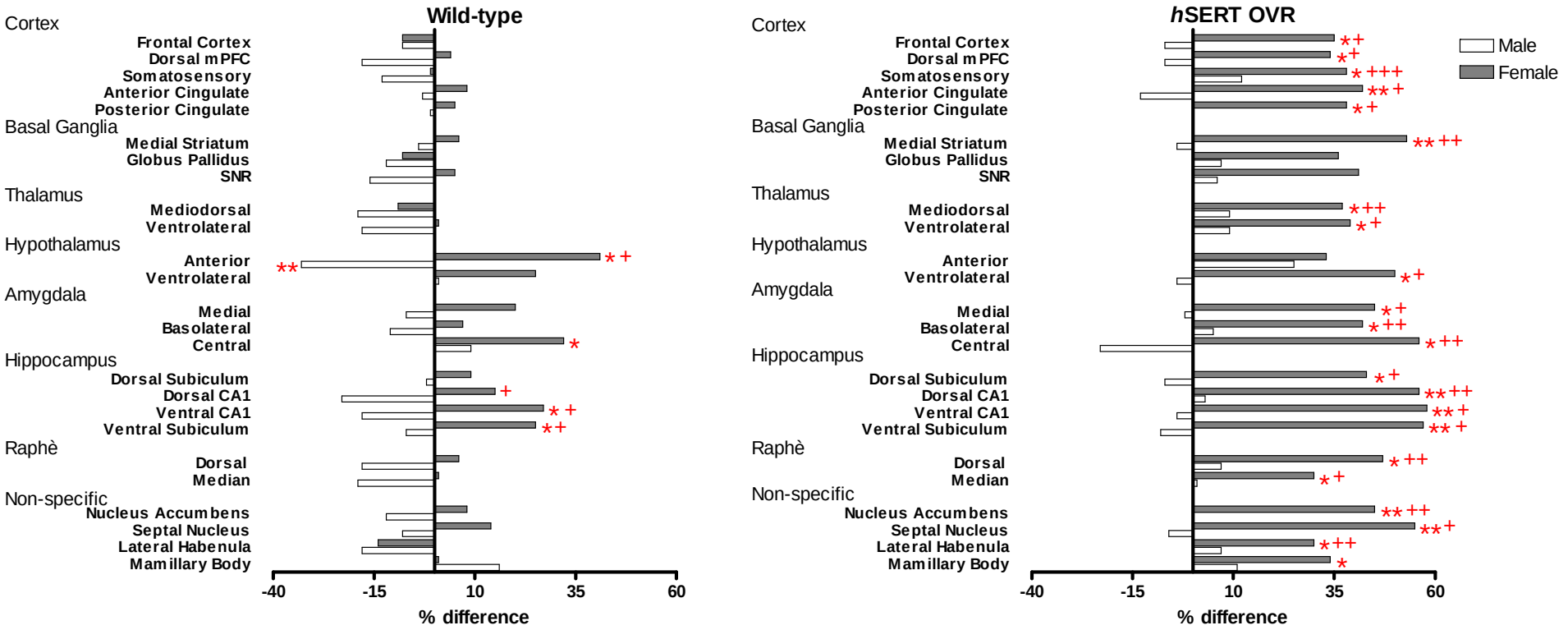
As the LCMRglu response to CP 94,253 was enhanced by *h*SERT OVR in females but not in males this meant that evidence for a significant gender x CP 94,253 interaction was more widespread in *h*SERT OVR as compared to Wt mice. This again suggests that modification of the response to CP 94,253 by *h*SERT OVR is influence by gender, but we found no significant evidence for this. Detailed data on the effects of *h*SERT over-expression on the LCMRglu response to CP 94,253 are shown in Tables 3.5.1 to 3.5.6. The plasma data for animals involved in this experiment are also shown in Appendix 1 (Table A1.4).

Figure 3.5.2 Effect of *h*SERT OVR on LCMRglu response to CP 94,253



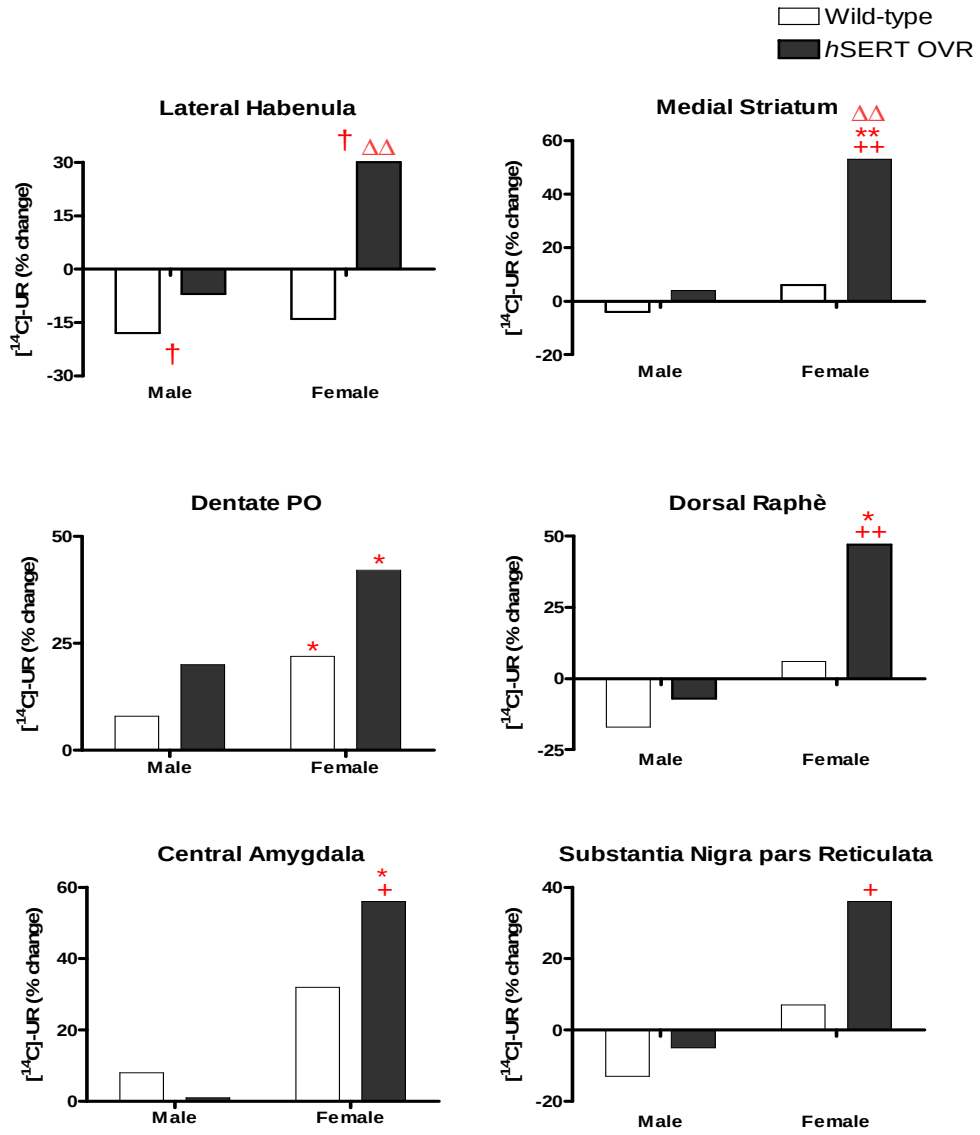
*Effect of hSERT over-expression on the LCMRglu ([^{14}C]-Uptake ratio; [^{14}C]-UR) response to 10 mg.kg^{-1} CP 94,253 in male and female mice. Data for 3 representative brain regions shown as mean ± s.e.m. *denotes p<0.05 significant hSERT effect (2-way ANOVA within saline animals with Bonferroni correction). $^{+}$denotes p<0.05 and $^{++}$denotes p<0.01 significant gender x CP 94,253 interaction (2-way ANOVA within genotype). †denotes significant hSERT OVR x CP 94,253 interaction. $^{\Delta}$denotes p<0.05 and $^{\Delta\Delta}$denotes p<0.01 significant CP 94,253 effect (2-way ANOVA within gender with Bonferroni correction). The LCMRglu response to CP 94,253 appears to be enhanced by hSERT over-expression in female mice but not in male mice. Unfortunately, the conservative nature of Univariate ANOVA means that a significant gender x hSERT OVR x CP 94,253 interaction was not found in any brain regions to support this suggestion.*

Figure 3.5.3 Gender differences in the LCMRglu response to CP 94,253



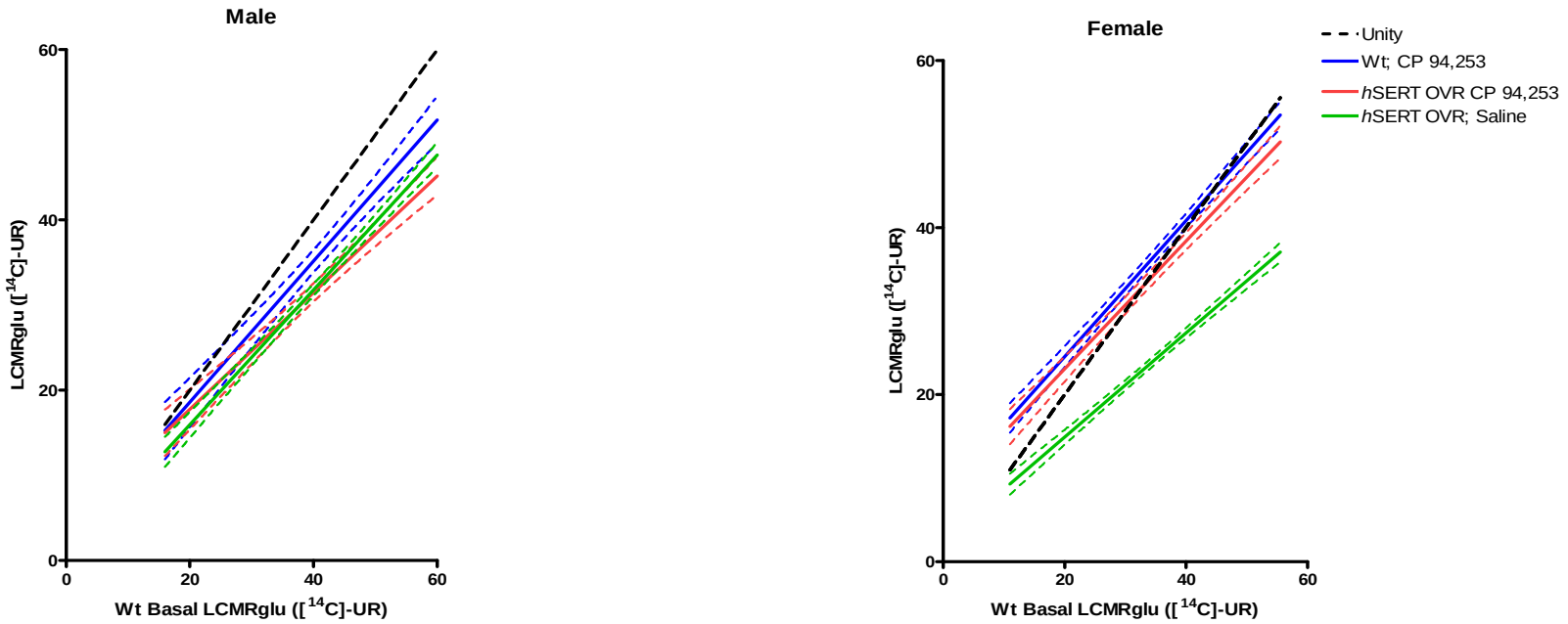
*Gender differences in the LCMRglu response to CP 94,253 in Wt and hSERT OVR mice. Data are shown as % difference in the [^{14}C]-Uptake ratio in CP 94,253-treated animals relative to the appropriate saline control and were analysed using 2-way ANOVA completed within genotype.$^{+}$denotes p<0.05, $^{++}$denotes p<0.01 and $^{+++}$denotes p<0.001 significant gender x CP 94,253 interaction. *denotes p<0.05 and **denotes p<0.01 significant CP 94,253 effect (with Bonferroni correction). Note in particular the enhancement of gender differences in the response to CP 94,253 in hSERT OVR as compared to Wt mice.*

Figure 3.5.4 Interaction between Gender and *h*SERT over-expression on the LCMRglu response to CP 94,253



*Effect of gender and hSERT OVR on the LCMRglu response to 10 mg.kg^{-1} CP 94,253 in male and female mice. Data for 6 representative brain regions shown as % change in the [^{14}C]-Uptake ratio ([^{14}C]-UR) between CP 94,253-treated animals and the appropriate saline control. $^{\Delta}$denotes p<0.05 and $^{\Delta\Delta}$denotes p<0.01 significant hSERT OVR x CP 94,253 interaction (2-way ANOVA within gender). *denotes p<0.05, **denotes p<0.01 significant CP 94,253 effect (2-way ANOVA with Bonferroni correction). $^{+}$denotes significant gender x CP 94,253 interaction and †denotes significant hSERT OVR x CP 94,253 interaction (Univariate ANOVA). The LCMRglu response to CP 94,253 was significantly greater in Wt females as compared to Wt males in a number of brain regions (e.g. dentate PO). This gender difference in the LCMRglu response to CP 94,253 is present in a greater number of brain regions in hSERT OVR mice (e.g. central amygdala, dorsal raphé). The response to CP 94,253 is enhanced in female hSERT over-expressing mice in several brain regions (e.g. medial striatum and lateral habenula).*

Figure 3.5.5 CP 94,253-treatment abolishes the cerebral hypo-metabolism observed in *h*SERT OVR female mice



Relationship between the Wt basal [^{14}C]-Uptake ratio ([^{14}C]-UR) and the [^{14}C]-UR in hSERT OVR and CP 94,256-treated animals across all brain regions analysed. Dashed black line representsthe level of Wt basal LCMRglu (Unity). Note in particular how hSERT OVR results in a pronounced hypo-metabolism throughout the brain in females (hSERT OVR; saline) as compared to Wt basal animals (unity line, saline treated) whereas in males (hSERT OVR;saline) this effect appears to be more modest. Also note how CP 94,256-treatment alleviates this hypo-metabolism in hSERT OVR females (hSERT OVR; CP 94.253), bringing the level of LCMRglu throughout the brain to the same level as observed in Wt animals (Wt;saline group represented by the unity line and the Wt; CP 94,253-treated group). Whereas the level of LCMRglu appears to be relatively unaffected by CP 94,253 treatment in males as the level of LCMRglu in hSERT OVR; CP 94,253 treated mice appears to be the same as that in hSERT OVR; saline treated mice.

Table 3.5.1 LCMRglu response to CP 94,253 in *h*SERT over-expressing mice: cortical regions

	Male						Female					
	Wild-type			hSERT OVR			Wild-type			hSERT OVR		
	Saline	CP 94,253	%	Saline	CP 94,253	%	Saline	CP 94,253	%	Saline	CP 94,253	%
Cortex												
Orbitofrontal	63 ± 6	54 ± 5	**-13**	46 ± 3	46 ± 3	**0**	49 ± 4	51 ± 2	**4**	35 ± 2$^{▲▲}$	46 ± 3	**33^{*++}**
Frontal	45 ± 5	41 ± 4	**-8**	33 ± 2	35 ± 2	**7**	44 ± 3	40 ± 2	**-8**	27 ± 2$^{▲▲▲}$	37 ± 1	**35$^{*\Phi+}$**
Anterior Cingulate	48 ± 5	46 ± 5	**-3**	35 ± 3	39 ± 2	**13**	43 ± 3	46 ± 2	**8**	30 ± 2$^{▲▲}$	42 ± 3	**42^{**+}**
Dorsal medial Prefrontal	47 ± 5	39 ± 4	**-18**	32 ± 2	34 ± 2	**7**	37 ± 3	38 ± 2	**4**	25 ± 2$^{▲▲}$	34 ± 2	**34^{*+}**
Ventral medial Prefrontal	41 ± 4	39 ± 4	**-17**	28 ± 2	28 ± 1	**0**	29 ± 2	31 ± 2	**8**	19 ± 2$^{▲▲}$	28 ± 1	**48^{*+++}**
Somatosensory	55 ± 4	47 ± 4	**-13**	44 ± 3	38 ± 2	**-12**	50 ± 5	49 ± 2	**-1**	31 ± 2$^{▲▲▲}$	42 ± 3	**38^{*+++}**
Temperoparietal	54 ± 5	43 ± 4	**-20**	43 ± 4	37 ± 2	**-14**	44 ± 4	44 ± 2	**0**	32 ± 2$^{▲}$	42 ± 5	**34^{++}**
Posterior Cingulate	52 ± 4	52 ± 6	**-1**	44 ± 3	44 ± 3	**0**	48 ± 4	51 ± 3	**5**	35 ± 2$^{▲}$	49 ± 5	**38^{*+}**
Piriform	31 ± 3	24 ± 3	**-21**	22 ± 1	22 ± 1	**0**	20 ± 2	24 ± 2	**24^{+}**	15 ± 5	23 ± 2	**51^{*++}**
Entorhinal	35 ± 3	31 ± 4	**-12**	28 ± 2	30 ± 1	**6**	30 ± 2	35 ± 2	**16**	22 ± 2$^{▲}$	32 ± 3	**45^{*+}**

Effect of *h*SERT over-expression on the LCMRglu response to CP 94,253 in cortical regions. Data shown as mean ± s.e.m. and % difference between CP 94,253-treated and control (saline-treated) animals of the same gender and genotype. *denotes p<0.05, **denotes p<0.01 and ***denotes p<0.001 significant CP 94,253 effect (within genotype, 2-way ANOVA with Bonferroni correction). $^{+}$denotes p<0.05, $^{++}$denotes p<0.05 and $^{+++}$denotes p<0.001 significant gender x CP 94,253 interaction (2-way ANOVA within genotype). $^{\Phi}$denotes p<0.05, $^{\Phi\Phi}$denotes p<0.01 significant genotype x CP 94,253 interactions (within gender 2-way ANOVA). †denotes p<0.05, ††denotes p<0.01 significant genotype x CP 94,253 interaction (Univariate ANOVA). $^{▲}$denotes p<0.05, $^{▲▲}$denotes p<0.01 significant *h*SERT effect (2-way ANOVA in gender).

Table 3.5.2 LCMRglu response to CP 94,253 in hSERT over-expressing mice: basal ganglia regions

	Male						Female					
	Wild-type			*h*SERT OVR			Wild-type			*h*SERT OVR		
	Saline	CP 94,253	%	Saline	CP 94,253	%	Saline	CP 94,253	%	Saline	CP 94,253	%
Basal Ganglia												
Medial Striatum	43 ± 3	41 ± 4	**-4**	35 ± 3	36 ± 2	**4**	42 ± 3	44 ± 2	**6**	28 ± 2$^{▲▲}$	43 ± 3	**53$^{**++Φ}$**
Lateral Striatum	48 ± 3	43 ± 4	**-12**	37 ± 3	37 ± 2	**-1**	42 ± 3	46 ± 3	**9**	30 ± 2$^{▲}$	45 ± 4	**51^{**++}**
Globus Pallidus	32 ± 2	28 ± 3	**-12**	26 ± 2	24 ± 1	**-7**	33 ± 3	30 ± 2	**-8**	21 ± 2$^{▲▲}$	28 ± 3	**36^{+}**
Subthalamic Nucleus	49 ± 5	42 ± 4	**-15**	43 ± 3	37 ± 2	**-15**	50 ± 3	45 ± 3	**-9**	34 ± 8	43 ± 4	**28 $^{Φ++}$**
Substantia Nigra pars Reticulata	30 ± 2	26 ± 2	**-13**	25 ± 2	24 ± 1	**-5**	26 ± 2	28 ± 2	**7**	19 ± 1$^{▲}$	26 ± 2	**36^{+}**
Substantia Nigra pars Compacta	38 ± 3	32 ± 3	**-16**	31 ± 2	29 ± 2	**-6**	34 ± 3	36 ± 2	**5**	24 ± 2$^{▲▲}$	33 ± 2	**41^{*++}**

Effect of *h*SERT over-expression on the LCMRglu response to CP 94,253 in cortical regions. Data shown as mean ± s.e.m. and % difference between CP 94,253-treated and control (saline-treated) animals of the same gender and genotype. *denotes p<0.05, **denotes p<0.01 and ***denotes p<0.001 significant CP 94,253 effect (within genotype, 2-way ANOVA with Bonferroni correction). $^{+}$denotes p<0.05, $^{++}$denotes p<0.05 and $^{+++}$denotes p<0.001 significant gender x CP 94,253 interaction (2-way ANOVA within genotype). †denotes p<0.05, ††denotes p<0.01 significant genotype x CP 94,253 interaction (Univariate ANOVA). ^{Φ}denotes p<0.05, ^{ΦΦ}denotes p<0.01 significant genotype x CP 94,253 interactions (within gender 2-way ANOVA). $^{▲}$denotes p<0.05, $^{▲▲}$denotes p<0.01 significant *h*SERT effect (2-way ANOVA in gender).

Table 3.5.3 LCMRglu response to CP 94,253 in *h*SERT over-expressing mice: amygdala, thalamic and hypothalamic regions

	Male						Female					
	Wild-type			hSERT OVR			Wild-type			hSERT OVR		
	Saline	CP 94,253	%	Saline	CP 94,253	%	Saline	CP 94,253	%	Saline	CP 94,253	%
Amygdala												
Medial	25 ± 2	23 ± 2	**-7**	19 ± 1	20 ± 1	**2**	18 ± 2	21 ± 2	**20**	14 ± 1	20 ± 2	**45*$^{+}$**
Basolateral	34 ± 3	30 ± 3	**-11**	28 ± 2	26 ± 1	**-5**	29 ± 2	31 ± 2	**7**	20 ± 2$^{▲}$	29 ± 3	**42*$^{++}$**
Central	23 ± 2	25 ± 2	**9**	18 ± 1	22 ± 1	**23**	17 ± 2	22 ± 2	**32***	13 ± 1	21 ± 2	**56*$^{++}$**
Thalamic Nuclei												
Anterior	50 ± 4	51 ± 5	**2**	46 ± 5	47 ± 3	**2**	47 ± 5	54 ± 3	**15**	36 ± 3	51 ± 5	**43*$^{++}$**
Mediodorsal	57 ± 5	47 ± 4	**-18**	45 ± 3	41 ± 2	**-9**	48 ± 4	48 ± 2	**1**	32 ± 2$^{▲▲}$	45 ± 4	**39*$^{++Φ}$**
Venterolateral	56 ± 5	45 ± 4	**-19**	47 ± 4	43 ± 3	**-9**	56 ± 5	51 ± 2	**-9**	34 ± 2$^{▲▲}$	46 ± 5	**37*$^{++ Φ}$**
Hypothalamic Nuclei												
Anterior	39 ± 5	26 ± 4	**-33****	30 ± 4	23 ± 1	**-25***	17 ± 1	24 ± 1	**41*$^{+}$**	14 ± 2$^{▲}$	19 ± 1	**33^{++}**
Venterolateral	30 ± 2	30 ± 3	**1**	26 ± 2	27 ± 2	**4**	26 ± 2	32 ± 3	**25**	18 ± 2$^{▲}$	27 ± 3	**50*$^{+}$**

Effect of *h*SERT over-expression on the LCMRglu response to CP 94,253 in cortical regions. Data shown as mean ± s.e.m. and % difference between CP 94,253-treated and control (saline-treated) animals of the same gender and genotype. *denotes p<0.05, **denotes p<0.01 and ***denotes p<0.001 significant CP 94,253 effect (within genotype, 2-way ANOVA with Bonferroni correction). $^{+}$denotes p<0.05, $^{++}$denotes p<0.05 and $^{+++}$denotes p<0.001 significant gender x CP 94,253 interaction (2-way ANOVA within genotype). †denotes p<0.05, ††denotes p<0.01 significant genotype x CP 94,253 interaction (Univariate ANOVA). ^{Φ}denotes p<0.05, ΦΦdenotes p<0.01 significant genotype x CP 94,253 interactions (within gender 2-way ANOVA). $^{▲}$denotes p<0.05, $^{▲▲}$denotes p<0.01 significant *h*SERT effect (2-way ANOVA in gender).

Table 3.5.4 LCMRglu response to CP 94,253 in *h*SERT over-expressing mice: hippocampal regions

	Male						Female					
	Wild-type			hSERT OVR			Wild-type			hSERT OVR		
	Saline	CP 94,253	%	Saline	CP 94,253	%	Saline	CP 94,253	%	Saline	CP 94,253	%
Hippocampus												
Molecular Layer	42 ± 4	34 ± 4	**-20**	32 ± 2	30 ± 2	**-7**	32 ± 3	36 ± 2	**11**$^{+}$	23 ± 2$^{\blacktriangle}$	33 ± 3	**42***$^{++}$
Dorsal Subiculum	40 ± 3	39 ± 4	**-2**	34 ± 3	36 ± 2	**7**	38 ± 3	41 ± 2	**9**	28 ± 2	40 ± 3	**43***$^{+}$
Dentate PO	25 ± 5	27 ± 3	**8**	21 ± 1	25 ± 1	**20**	20 ± 2	24 ± 2	**22***	17 ± 2	24 ± 2	**42***
Dorsal CA1	37 ± 3	29 ± 3	**-23**	29 ± 2	28 ± 2	**-3**	28 ± 3	33 ± 2	**15**$^{++}$	19 ± 2$^{\blacktriangle}$	30 ± 2	**56****$^{++}$
CA2	35 ± 3	29 ± 3	**-17**	26 ± 2	27 ± 4	**8**	26 ± 3	30 ± 2	**17**$^{+}$	18 ± 2$^{\blacktriangle}$	29 ± 3	**61****$^{+}$
Ventral CA1	34 ± 2	28 ± 3	**-18**	26 ± 2	27 ± 2	**4**	24 ± 2	31 ± 2	**27***$^{++}$	19 ± 1	30 ± 3	**58****$^{+}$
Ventral Subiculum	30 ± 2	28 ± 3	**-7**	24 ± 2	26 ± 2	**8**	23 ± 2	29 ± 2	**25***$^{+}$	19 ± 2	30 ± 4	**57****$^{+}$
CA3	28 ± 2	27 ± 3	**-5**	22 ± 1	24 ± 1	**11**	21 ± 2	26 ± 2	**25***	15 ± 1	24 ± 2	**53***$^{+}$

Effect of *h*SERT over-expression on the LCMRglu response to CP 94,253 in cortical regions. Data shown as mean ± s.e.m. and % difference between CP 94,253-treated and control (saline-treated) animals of the same gender and genotype. *denotes p<0.05, **denotes p<0.01 and ***denotes p<0.001 significant CP 94,253 effect (within genotype, 2-way ANOVA with Bonferroni correction). $^{+}$denotes p<0.05, $^{++}$denotes p<0.05 and $^{+++}$denotes p<0.001 significant gender x CP 94,253 interaction (2-way ANOVA within genotype). †denotes p<0.05, ††denotes p<0.01 significant genotype x CP 94,253 interaction (Univariate ANOVA). $^{\Phi}$denotes p<0.05, ΦΦdenotes p<0.01 significant genotype x CP 94,253 interactions (within gender 2-way ANOVA). $^{\blacktriangle}$denotes p<0.05, $^{\blacktriangle\blacktriangle}$denotes p<0.01 significant *h*SERT effect (2-way ANOVA in gender).

Table 3.5.5 LCMRglu response to CP 94,253 in *h*SERT over-expressing mice: raphé and mesocorticolimbic regions

	Male						Female					
	Wild-type			hSERT OVR			Wild-type			hSERT OVR		
	Saline	CP 94,253	%	Saline	CP 94,253	%	Saline	CP 94,253	%	Saline	CP 94,253	%
Raphé												
Dorsal	38 ± 3	32 ± 2	**-18**	30 ± 2	28 ± 1	**-7**	29 ± 2	30 ± 2	**6**	20 ± 2$^{\blacktriangle}$	30 ± 2	**47*$^{++}$**
Median	47 ± 5	39 ± 3	**-19**	37 ± 3	37 ± 2	**-1**	39 ± 3	39 ± 2	**1**	28 ± 2$^{\blacktriangle}$	36 ± 3	**30^{+}**
Paramedian	47 ± 4	39 ± 3	**-17**	37 ± 3	36 ± 2	**-1**	40 ± 3	40 ± 2	**1**	28 ± 2$^{\blacktriangle}$	39 ± 2	**38*$^{+}$**
Mesocorticolimbic System												
Ventral Tegmental Area	38 ± 3	41 ± 3	**6**	36 ± 4	40 ± 3	**11**	43 ± 4	44 ± 3	**4**	31 ± 23	48 ± 5	**56*$^{+}$**
Nucleus Accumbens	43 ± 6	38 ± 3	**-12**	31 ± 2	31 ± 1	**0**	31 ± 2	34 ± 2	**8**	21 ± 2$^{\blacktriangle\blacktriangle}$	31 ± 2	**45**$^{++}$**

Effect of *h*SERT over-expression on the LCMRglu response to CP 94,253 in raphé, mesocorticolimbic and non-specific regions. Data shown as mean ± s.e.m. and % difference between CP 94,253-treated and control (saline-treated) animals of the same gender and genotype. *denotes p<0.05, **denotes p<0.01 and ***denotes p<0.001 significant CP 94,253 effect (within genotype, 2-way ANOVA with Bonferroni correction). +denotes p<0.05, ++denotes p<0.05 and +++denotes p<0.001 significant gender x CP 94,253 interaction. †denotes p<0.05, ††denotes p<0.01 significant genotype x CP 94,253 interaction (Univariate ANOVA). Φdenotes p<0.05, ΦΦdenotes p<0.01 significant genotype x CP 94,253 interactions (within gender 2-way ANOVA). $^{\blacktriangle}$denotes p<0.05, $^{\blacktriangle\blacktriangle}$denotes p<0.01 significant *h*SERT effect (2-way ANOVA in gender).

Table 3.5.6 LCMRglu response to CP 94,253 in *h*SERT over-expressing mice: non-specific regions

	Male						Female					
	Wild-type			hSERT OVR			Wild-type			hSERT OVR		
	Saline	CP 94,253	%	Saline	CP 94,253	%	Saline	CP 94,253	%	Saline	CP 94,253	%
Non-specific												
Septal Nucleus	35 ± 3	32 ± 3	**-8**	28 ± 2	30 ± 2	**6**	29 ± 2	33 ± 2	**14**	21 ± 2$^{\blacktriangle}$	32 ± 3	**55**$^{+}$**
Bed Nucleus of the Stria Terminalis	27 ± 3	21 ± 2	**-21**	20 ± 1	20 ± 1	**0**	17 ± 2	21 ± 2	**25^{+}**	12 ± 2$^{\blacktriangle}$	21 ± 1	**68*$^{++}$**
Corpus Callosum	16 ± 1	15 ± 2	**-8**	13 ± 1	13 ± 1	**4**	11 ± 2	15 ± 2	**36^{+}**	8 ± 1	13 ± 1	**52^{+}**
Lateral Habenula	56 ± 5	46 ± 4	**-18**	44 ± 3	41 ± 2	**-7**	55 ± 4	47 ± 2	**-14**	36 ± 2$^{\blacktriangle\blacktriangle}$	47 ± 4	**30*$^{++\Phi\Phi}$**
Mamillary Body	55 ± 4	64 ± 7	**16**	46 ± 3	51 ± 4	**11**	56 ± 12	56 ± 4	**1**	38 ± 2	51 ± 5	**56*$^{+\Phi}$**
Periaqueductal Grey	29 ± 4	27 ± 2	**-7**	25 ± 1	25 ± 1	**0**	23 ± 2	26 ± 2	**13**	17 ± 2	26 ± 2	**47*$^{+}$**
Inferior Colliculus	72 ± 8	69 ± 10	**-4**	75 ± 8	68 ± 5	**-9**	81 ± 7	80 ± 4	**-2**	54 ± 4$^{\blacktriangle}$	73 ± 6	**35*$^{+}$**
Ventral Tegmental Nucleus	51 ± 4	42 ± 4	**-17**	39 ± 3	38 ± 3	**-3**	43 ± 3	43 ± 2	**-2**	29 ± 2$^{\blacktriangle}$	42 ± 4	**42^{+}**
Locus Coeruleus	44 ± 4	34 ± 3	**-23**	35 ± 3	35 ± 2	**1**	38 ± 3	36 ± 3	**-4**	25 ± 2$^{\blacktriangle}$	31 ± 2	**24**
Nucleus Tractus Solitaris	49 ± 7	31 ± 2	**-36**	40 ± 4	30 ± 2	**-26**	37 ± 4	33 ± 2	**-11**	18 ± 1	29 ± 3	**62**

Effect of *h*SERT over-expression on the LCMRglu response to CP 94,253 in raphé, mesocorticolimbic and non-specific regions. Data shown as mean ± s.e.m. and % difference between CP 94,253-treated and control (saline-treated) animals of the same gender and genotype. *denotes p<0.05, **denotes p<0.01 and ***denotes p<0.001 significant CP 94,253 effect (within genotype, 2-way ANOVA with Bonferroni correction). +denotes p<0.05, ++denotes p<0.05 and +++denotes p<0.001 significant gender x CP 94,253 interaction. †denotes p<0.05, ††denotes p<0.01 significant genotype x CP 94,253 interaction (Univariate ANOVA). $^{\Phi}$denotes p<0.05, $^{\Phi\Phi}$denotes p<0.01 significant genotype x CP 94,253 interactions (within gender 2-way ANOVA). $^{\blacktriangle}$denotes p<0.05, $^{\blacktriangle\blacktriangle}$denotes p<0.01 significant *h*SERT effect (2-way ANOVA in gender).

5.4 Discussion and Conclusions

In contrast to other studies reporting on the ability of genetically altered SERT function to alter 5-HT_{1B} binding, specifically in the substantia nigra of SERT KO animals (Fabre et al., 2000b), we found no evidence for altered 5-HT_{1B} binding in this region or others (ventral pallidum and globus pallidus) in *h*SERT OVR mice. This may directly relate to the different magnitudes of altered SERT functioning present in these different animal models. Regulation of extracellular 5-HT levels by SERT function likely represents a key mechanism by which SERT function regulates 5-HT_{1B} receptor expression in SERT KO animals, especially of the post-synaptic 5-HT_{1B} heteroreceptors. In agreement with our finding others have reported on the inability of chronic alterations in extracellular 5-HT levels to alter 5-HT_{1B} expression (Le Poul et al., 2000). However, as we could only determine 5-HT_{1B} binding in a limited number of brain regions, those which express particularly high levels of the receptor, it is possible that 5-HT_{1B} receptor binding may be altered in brain regions not measured in our study. Furthermore, the conditions used in this study are likely to detect binding not only at the 5-HT_{1B} receptor but also at the 5-HT_{1D} receptor as [^{3}H]GR 125,743 has affinity for both of these binding sites and the concentration of CP 93,129 used for the determination of NSB binding in these studies is likely to occupy a significant percentage of both of these receptor subtypes (Bruinvels et al., 1993). Therefore, it is possible that any alteration in 5-HT_{1B} binding may have been masked by 5-HT_{1D} binding. This suggestion, along with the fact that only high levels of 5-HT_{1B} receptor binding are detected using the present methodology, suggests that further optimisation of the ligand binding protocol used in this study is necessary before definitive conclusions on 5-HT_{1B} binding can be made in *h*SERT OVR animals. However, overall our data suggest that 5-$HT_{1B/1D}$ receptor binding is not altered by *h*SERT OVR in those 3 brain regions measured in our analysis.

In this study administration of the selective 5-HT_{1B} agonist CP 94,253 resulted in an increase in cerebral metabolism that was particularly widespread within the hippocampus and also present in the central amygdala of Wt female animals. In contrast, CP94,253 did not increase cerebral metabolism in Wt males. Infact, a generalised trend towards decreased metabolism was observed in Wt males but this only reached significance in the anterior hypothalamus. Only one other study has reported on the cerebral metabolic impact of 5-HT_{1B} receptor activation (Kelly et al., 1988) and the results in our study stand in stark contrast to those previously reported. For example, Kelly et al. (1988) found no evidence for significantly altered metabolism in the anterior hypothalamus. Furthermore, significant increases in LCMRglu were detected in numerous brain regions (including cortical and basal ganglia structures) that were not found in our study. One reason for these disparities could be the use

of the highly selective 5-HT_{1B} agonist CP 94,253 in this study as compared to use of the non-selective 5-HT_{1B} agonist RU 24,969, which also possesses 5-$HT_{2A/C}$ affinity. However, as the published study also involved measurement of LCMRglu in a greater number of brain areas (72), many of which were not included in our analysis, a more comprehensive analysis may reveal greater similarity between these studies.

Our results suggest that use of the 2-DG method to determine the precise localisation of altered 5-HT_{1B} receptor function in the CNS may not be as appropriate as originally thought. The regional distribution of LCMRglu responses to CP 94,253 does not accurately reflect that of 5-HT_{1B} receptor distribution. Furthermore, observations of increased LCMRglu in response to CP 94,253 are unlikely to reflect the activation of 5-HT_{1B} receptors localised within these areas. Activation of terminal 5-HT_{1B} receptors inhibits neurotransmitter release and neuronal activity which would decrease metabolic demand at the synapse. Therefore, decreases in LCMRglu would be expected to occur as a result of the 5-HT_{1B} receptor activation in each brain region. It is likely that increases in LCMRglu following CP 94,253 administration reflect the complex integration of decreases in the metabolic demand of nerve terminals possessing 5-HT_{1B} receptors in each region, alterations in the synaptic activity of inputs into that region (which may be modified by "up-stream" 5-HT_{1B} receptor activation) and alterations in neuronal activity that result from complex alterations in the availability multiple neurotransmitters.

In this study a significant sexually dimorphic LCMRglu response to CP 94,253 was noted in multiple regions of the hippocampus, the central amygdala and the anterior hypothalamus of Wt animals. In the hippocampus and central amygdala this resulted from a significant increase in metabolism in females but not in males. It is difficult to determine what this dimorphic response means in terms of gender differences in basal 5-HT_{1B} functioning, in part due to the vast difference in base-line LCMRglu between genders. The results may be taken to suggest that the 5-HT_{1B} receptors responsible for these responses display lower functional activity in males than in females. On the other hand it may be suggested that the level of functioning of these 5-HT_{1B} receptors is not different between the genders but that they are tonically activated in males, therefore application of the agonist elicits no response, but in females they are not. Indeed, there is some evidence to support the tonic activation of both 5-HT_{1B} autoreceptors (Kikvadze and Foster, 1995) and heteroreceptors (Querejeta et al., 2005) in selected brain regions under basal conditions. Furthermore, the increased SERT expression (study 1), consistent with enhanced 5-HT re-uptake, and the decreased level of extracellular 5-HT (Jones and Lucki, 2005; Mitsushima et al., 2006) found in females as compared to males would be consistent with reduced tonic activation of 5-HT_{1B} receptors in females under

basal conditions (if 5-HT_{1B} receptor functional levels were the same between genders). Furthermore, 5-HT_{1B} hypostimulation in females as compared to males as a result of decreased synaptic 5-HT availability would be consistent with the enhancement of gender differences in the response to CP 94,253 by *h*SERT OVR (see later in discussion). However, definitive conclusions upon the basal activation of 5-HT_{1B} receptors between genders can not be made on the basis of our evidence and further research is required. To further clarify this issue it may be of particular interest to characterise dose-response curves for LCMRglu following 5-HT_{1B} antagonist application in males and females. If the 5-HT_{1B} receptors responsible for these LCMRglu responses are tonically active in males but not in females one may expect to find a shift to the left (lower dose required to elicit response) in the LCMRglu dose-response curve of 5-HT_{1B} antagonists in males as compared to females. If the activation of these receptors between genders was not different under basal conditions one would expect to see no difference in the dose-response curve. Whereas, if 5-HT_{1B} function is greater in females than in males one may expect to see the dose-response curve of females shifted to the left to that of males.

The most profound gender difference in the response to CP 94,253 was noted in the anterior hypothalamus where a significant decrease was observed in males (Wt -35%, hSERT OVR -25%) and significant increases were observed in females (Wt +41%, hSERT OVR +33%). The limited knowledge on the gender-dependent effects of 5-HT_{1B} receptor activation makes identifying the possible mechanisms involved in this dramatic gender difference difficult to identify.

Despite the increased amplitude of the cerebral metabolic response to CP 94,253 in *h*SERT OVR females as compared to Wt females in a number of brain regions (Figure 3.5.3) we suggest that our data do *not* support our original hypothesis; that 5-HT_{1B} receptor function is increased in *h*SERT OVR mice. Rather, we suggest that our data support the contention that 5-HT_{1B} receptor signalling is hypoactive in *h*SERT OVR females under basal conditions and that this contributes to the constitutive hypo-metabolism observed in these animals.

In females the LCMRglu response to CP 94,253 was significantly increased in 5 brain regions in *h*SERT OVR mice as compared to Wt animals. These regions included the frontal cortex, medial striatum, subthalamic nucleus, ventrolateral and mediodorsal thalamus and the lateral habenula. Each of these regions displayed a significant basal hypo-metabolism in *h*SERT OVR females as compared to Wt females. This hypo-metabolism was abolished in *h*SERT OVR mice by treatment with the 5-HT_{1B} agonist CP 94,253, bringing LCMRglu in these regions to a level comparable to that observed in Wt animals (Figure 3.5.2 provides

representative examples). In Wt females CP 94,253 failed to elicit a significant LCMRglu response in any of these regions. This suggests that the 5-HT_{1B} receptors responsible for these responses are tonically activated in Wt female animals but are hypo-stimulated in female *h*SERT OVR animals. The normalisation of the basal hypo-metabolism in female *h*SERT OVR animals by CP 94,253 treatment to a level comparable to that of Wt animals was a trend observed throughout the entire brain (Figure 3.5.5). This suggests that 5-HT_{1B} hypo-stimulation in *h*SERT OVR females directly contributes to the basal hypo-metabolism observed in the limbic system of these animals. There was no evidence for 5-HT_{1B} hypo-stimulation in male *h*SERT OVR mice as there was no evidence for basal hypo-metabolism or an altered response to CP 94,253 in male *h*SERT OVR as compared to Wt male mice. Unfortunately, however, lack of a significant gender x genotype x CP 94,253 interaction in any brain region meant that the modulatory influence of gender on *h*SERT OVR induced 5-HT_{1B} receptor hypo-stimulation was not confirmed.

Hypo-stimulation of 5-HT_{1B} receptors in *h*SERT OVR females may result from a lack of a compensatory alteration in 5-HT_{1B} receptor function to the decreased levels of extracellular 5-HT in these animals. Lack of evidence for the hypo-stimulation of 5-HT_{1B} receptors in male mice suggests that compensatory alterations in 5-HT_{1B} receptor functioning may occur in male *h*SERT OVR mice. Alternatively, females may be more sensitive to decreases in 5-HT_{1B} receptor activation than males as they may be closer to the threshold level of 5-HT_{1B} stimulation at which alterations in LCMRglu are observed. This suggestion is supported by observations of the decreased extracellular 5-HT availability in females and possibly by our own observations of an LCMRglu response to CP 94,253 in Wt females but not Wt males. This would mean that alterations in 5-HT availability in *h*SERT OVR mice may result in significant 5-HT_{1B} hypo-stimulation in females but not in males. On the other hand this gender difference may be a direct result of the greater enhancement of SERT expression by *h*SERT OVR in females as compared to males (study 1). This would suggest that synaptic 5-HT levels may be decreased to a greater extent in *h*SERT OVR females in comparison to males in some brain regions. Therefore, the theoretical threshold at which 5-HT_{1B} receptors become significantly hypo-simulated in *h*SERT OVR mice may be achieved in these regions in females but not in males.

Unfortunately use of the 2-deoxglucose technique to assess 5-HT_{1B} receptor function does not allow us to determine the localisation of the 5-HT_{1B} receptors or the relative importance of the autoreceptor versus the heteroreceptor in the basal hypo-metabolism observed in *h*SERT OVR females. However, as enhancement of 5-HT neurotransmission has been shown to stimulate metabolism in many of the brain regions which display hypo-metabolism in female

*h*SERT OVR mice (Bremner et al., 1997; Cudennec et al., 1988b; Mann et al., 1996) it seems unlikely that 5-HT_{1B} autoreceptor activation, which would further reduced synaptic 5-HT, would be responsible for the increase in metabolism in these regions following CP 94,253 administration. Therefore, we suggest that hypo-stimulation of 5-HT_{1B} heteroreceptors may be involved in the hypo-metabolism observed in *h*SERT OVR females. In particular hypo-activation of 5-HT_{1B} heteroreceptors localised on GABAergic neurones may be particularly important. 5-HT_{1B} heteroreceptor activation decreases the availability of multiple neurotransmitters including glutamate (Boeijinga and Boddeke, 1996), acetylcholine (Maura and Raiteri, 1986) and GABA (Bramley et al., 2005; Johnson et al., 1992; Stanford and Lacey, 1996). As acetylcholine and glutamate are classically excitatory neurotransmitters it seems unlikely that the dis-inhibition of cholinergic or glutamatergic neurotransmission (by reduced 5-HT_{1B} heteroreceptor activation) could account for the cerebral hypo-metabolism in *h*SERT OVR females. Indeed, treatment with acetylcholine (nicotinic and muscarinic) receptor antagonists produces widespread increases in cerebral metabolism (Dowedwards et al., 1981; Grunwald et al., 1988; London et al., 1988). Furthermore, glutamate receptor activation is associated with widespread increases in cerebral metabolism (Fowler et al., 2004; Jordan et al., 2005) whereas decreases in glutamatergic neurotransmission (MacKay et al., 1994) and glutamate receptor antagonists (Browne and McCulloch, 1994) reduce cerebral metabolism. As the primary inhibitory neurotransmitter in the CNS it seems more likely that the dis-inhibition of GABAergic neurotransmission (by reduced 5-HT_{1B} stimulation) may contribute to the cerebral hypometabolism in *h*SERT OVR females. Indeed, there are striking parallels between the pattern of hypo-metabolism observed in *h*SERT OVR female mice and that induced by GABA receptor agonists (Study 2 discussion; (Cudennec et al., 1987; Kelly and McCulloch, 1982; Kelly and MCculloch, 1983b). Furthermore, evidence from 5-HT_{1B} mice supports the suggestion that 5-HT_{1B} heteroreceptors tonically inhibit GABAergic neurotransmission, at least in the suprachiasmatic nucleus (Bramley et al., 2005). The disinhibition of GABAergic neurotransmission by reduced 5-HT_{1B} heteroreceptor activation may represent a common mechanisms contributing to decreased anxiety-like behaviour in both 5-HT_{1B} KO (Brunner et al., 1999; Zhaung et al., 1999; Mallaret et al., 1999) and hSERT OVR mice (Jennings et al., 2006). Clearly, the possible interaction between 5-HT_{1B} hypo-stimulation and the function of other neurotransmitter systems in determination of the cerebral hypo-metabolism found in *h*SERT OVR mice warrants further investigation. Utilisation of *in vivo* microdialysis may be particularly useful in assessing these interactions and the data from these 2-DG experiments provides a guide to those regions in the CNS which may be of particular interest. Measuring neurotransmitter levels under basal conditions and in response to application of 5-HT_{1B} agonists would allow the assessment of 5-HT_{1B}

autoreceptor (5-HT detection) and heteroreceptor (glutamate, acetylcholine, GABA detection) function in female *h*SERT OVR mice.

To summarise, our data suggest that 5-$HT_{1B/1D}$ receptor binding is not influence by gender or by a life-long increase in SERT functioning, at least in a limited number of brain areas. In contrast, functional responses to 5-HT_{1B} agonists are greater in females than in males although it is unclear as to whether this reflects a gender difference in 5-HT_{1B} activation under basal conditions. Furthermore, 5-HT_{1B} receptors appear to be hypo-stimulated as a result of a life-long increase in SERT functioning in females and this may contribute to the constitutive decrease in cerebral metabolism noted in these animals. Hypo-stimulation of 5-HT_{1B} receptors does not appear to result from a life-long increase in SERT functioning in males, which parallels the observation of unaltered constitutive cerebral metabolism in *h*SERT OVR males.

Study 6- Hypothalamo-pituitary-adrenal axis function in *h*SERT OVR mice

6.1 Rationale

The functional activity of the HPA axis has been proposed to have a central role in the aetiology of affective disorders and in influencing the efficacy of antidepressant treatment. Furthermore, there is a wide body of evidence supporting reciprocal interaction between the 5-HT system and the HPA axis under both normal conditions and in disease states. Given the widespread evidence for altered 5-HT system functioning in *h*SERT OVR mice (studies 2-5) the possibility that HPA axis activity may also be altered in these animals is of considerable interest. In particular, the integration of the 5-HT system and HPA axis in these animals, where there is a life-long enhancement of SERT function, may be particularly relevant to the known influence of the 5-HTTLPR on the likelihood of developing affective disorders in response to stressful life events. In humans the "long" allele of the 5-HTTLPR is associated with a decreased risk in the development of affective disorders. In contrast, individuals who carry the "short" allele are more susceptible to the negative effects of stressful life-events (Caspi et al., 2003; Kendler et al., 2005). Thus, differential integration between central serotonergic systems and the HPA axis between these genotypes, and resultant changes to stress responses, may represent one mechanism by which individuals may be differentially predisposed to the development of affective disorders. Indeed, evidence from animal studies suggests that genetically-determined levels of SERT expression do indeed govern HPA axis activity during stressful events (Barr et al., 2004b; Li et al., 1999).

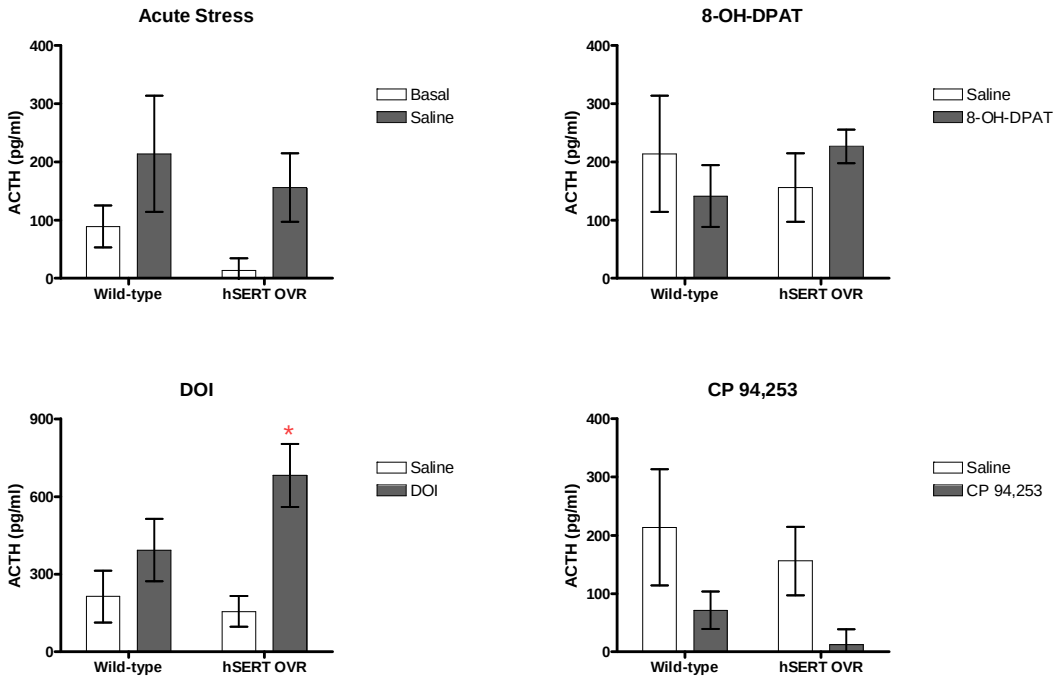
In order to further understand the possible influence of a life-long increase in SERT expression on HPA axis function, circulating ACTH and corticosterone levels were measured in the following studies under basal conditions (rapid decapitation) and in response to acute mild stress (saline injection 15 minutes prior to rapid decapitation) in male Wt and *h*SERT OVR mice. Furthermore, to identify possible alterations in the serotonergic feed-forward control of the HPA axis in *h*SERT OVR mice these hormone levels were measured following challenge with specific 5-HT receptor agonists (namely 8-OH-DPAT for 5-HT_{1A}, DOI for 5-$HT_{2A/C}$ and CP 94,253 for 5-HT_{1B} receptors). Finally, to define possible alterations in feed-back mechanisms regulating HPA axis functioning, GR and MR mRNA expression levels were measured by in situ hybridisation in areas of the CNS known to be associated with the negative feedback control of the HPA axis by circulating glucocorticoids.

6.2 Circulating stress hormones

Under basal conditions there was no evidence for a significant difference in the level of circulating ACTH or corticosterone between Wt and *h*SERT OVR mice. Acute mild stress (saline injection, 0.2ml) produced a significant increase in circulating corticosterone in animals of both genotypes (Wt 2.8-fold, *h*SERT OVR 2.5-fold). Despite a trend towards increased ACTH levels in animals of both genotypes following acute stress, the effect was not significant. This was possibly due to high variability in the levels of ACTH detected in saline-treated mice, especially in Wt animals. Circulating levels of ACTH were not altered by 8-OH-DPAT (1.0 $mg.kg^{-1}$) in animals of either genotype when these were compared to saline injected controls. In contrast, circulating levels of corticosterone were significantly elevated in both Wt and *h*SERT OVR mice in response to the same 8-OH-DPAT treatment (Wt 2-fold increase, *h*SERT OVR 1.5-fold increase over saline injected controls), but there was no significant difference in the hormonal response to 8-OH-DPAT between the two genotypes.

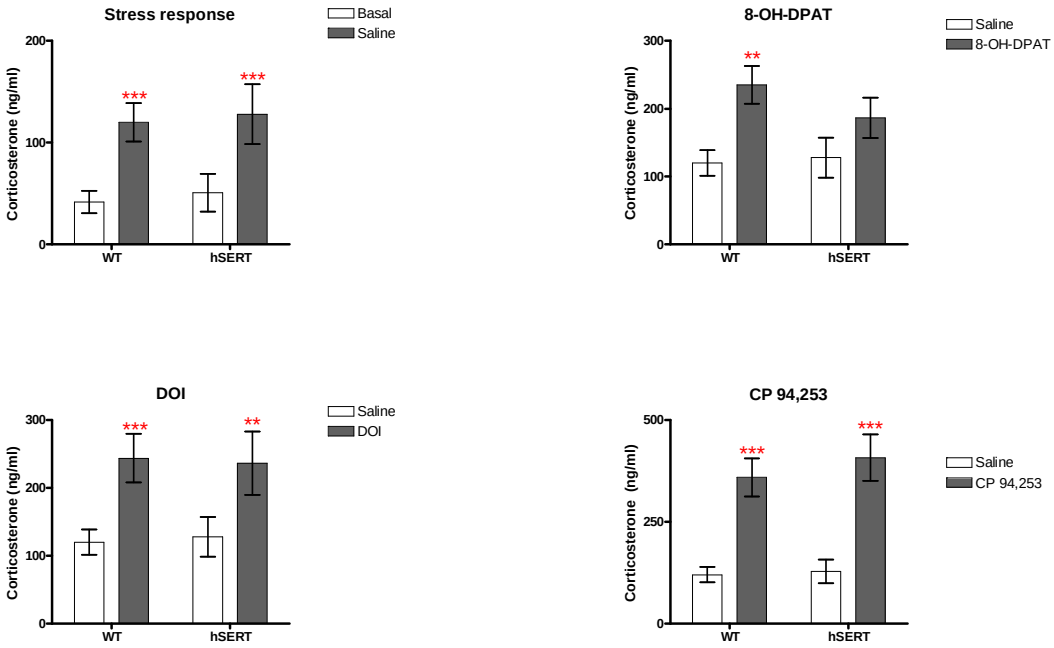
DOI treatment (2.5 $mg.kg^{-1}$) produced a significant elevation in plasma ACTH in animals of both genotypes (Wt 1.6-fold increase, *h*SERT 4.4-fold increase) and also resulted in a significant increase in corticosterone (Wt 2.0-fold increase, *h*SERT 1.8-fold increase). Although the magnitude of the ACTH response to DOI appeared to be greater in *h*SERT OVR, again there was no evidence for a significant difference in the hormonal response to DOI between genotypes. CP 94,253-treatment (10$mg.kg^{-1}$) produced a trend towards decreased ACTH levels in both genotypes in comparison to saline treated animals, but this effect was not significant. In contrast, CP 94,253-treatment resulted in a marked elevation of circulating corticosterone (3-fold increase) in both genotypes. There was no evidence for a significant difference in the hormonal response to CP 94,253 between genotypes. All ACTH and corticosterone data are shown in Figures 3.6.1 and 3.6.2 respectively.

Figure 3.6.1 ACTH responses in *h*SERT OVR mice



*ACTH levels in Wt and hSERT OVR mice under basal conditions, following acute mild stress (saline injection) and treatment with the 5-HT receptor agonists 8-OH-DPAT (1.0 mg.kg^{-1}), DOI (2.5 mg.kg^{-1}) and CP 94,253(10 mg.kg^{-1}). *denotes p<0.05 significant drug effect (2-WAY ANOVA with Bonferroni correction). There was no evidence for a significant difference in basal ACTH or in the alteration of ACTH secretion following mild stress or 5-HT receptor agonist treatment between the different genotypes.*

Figure 3.6.2 Corticosterone responses in *h*SERT OVR mice



*Corticosterone levels in Wt and hSERT OVR mice under basal conditions, following acute mild stress (saline injection) and treatment with the 5-HT receptor agonists 8-OH-DPAT (1 mg.kg^{-1}), DOI (2.5 mg.kg^{-1}) and CP 94,253 (10 mg.kg^{-1}). ***denotes p<0.01 significant drug effect (2-WAY ANOVA with Bonferroni correction). There was no significant evidence for a difference between the genotypes in basal corticosterone levels, the corticosterone response to mild acute stress or in the response to any of the different 5-HT receptor agonists.*

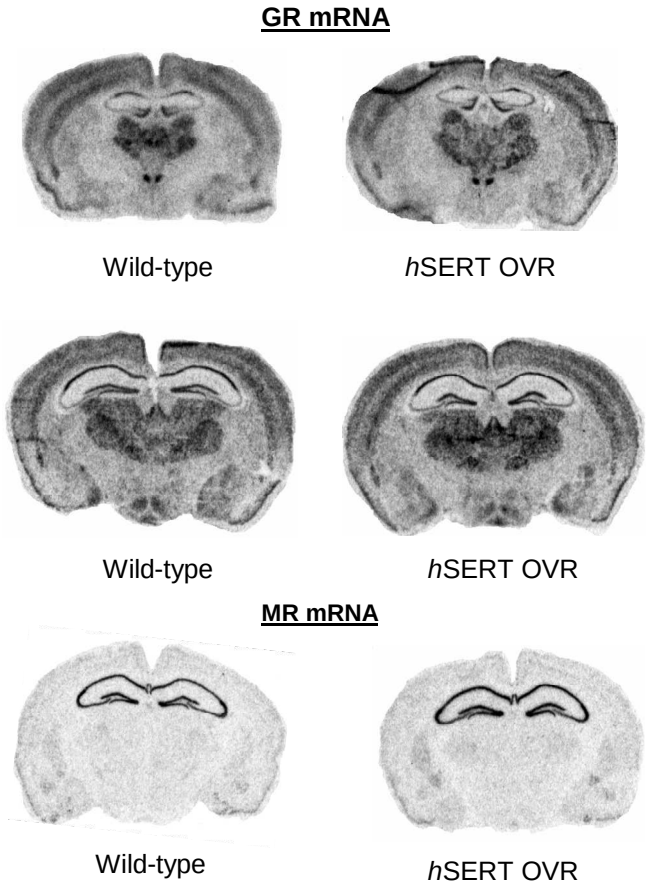
6.3 Glucocorticoid and mineralocorticoid receptor mRNA expression

The distribution and relative density of GR mRNA expression detected throughout the brain in this study was consistent with that previously reported (Chao et al., 1989; Morimoto et al., 1996; Sousa et al., 1989). GR mRNA expression was highest in the paraventricular nucleus (PVN) of the hypothalamus and high levels were also present in subfields of the hippocampus (CA1, CA2, DG). More moderate expression was noted in neocortex and thalamus, while low levels of expression were detected in the amygdala nuclei and CA3 and CA4 subfields of the hippocampus (Figure 3.6.3). The level of GR mRNA expression was found to be significantly increased in *h*SERT OVR as compared to Wt mice in only one brain region, the PVN (Figure 3.6.5). In all other brain regions analysed there was no evidence for a significant difference, or even any trend towards difference, in the level of GR mRNA expression between the different genotypes.

The distribution and relative densities of MR mRNA expression detected throughout the brain in this study were also consistent with those previously reported (Chao et al., 1989). MR mRNA expression was highest in CA3 and DG fields of the hippocampus. Moderate levels of expression were detected in other hippocampal subfields (CA1, CA2, CA4) while very low levels of expression were found in the amygdala and ventrolateral thalamus (Figure 3.6.3). The density of MR mRNA was significantly increased in CA1 and CA2 subfields of the hippocampus in *h*SERT OVR mice (Figure 3.6.4). In all other brain regions analysed there was no evidence for a significant difference in MR mRNA expression between *h*SERT OVR and Wt mice. Detailed data for both MR and GR in situ hybridisation are shown in Tables 3.6.1. and 3.6.2.

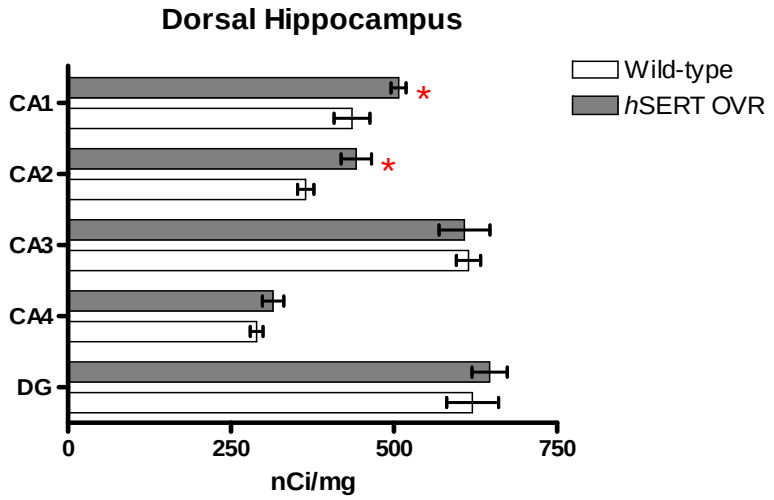
The specificity of both the GR and MR 'anti-sense' RNA probes was demonstrated using the appropriate ^{35}S-labelled 'sense' probe of similar specific activity, hybridized under identical conditions. No specific hybridization signal was detected with either the GR or MR 'sense' probes and the level of radioactivity in each brain region in 'sense' hybridized sections was indistinguishable from background.

Figure 3.6.3 GR and MR mRNA autoradiograms



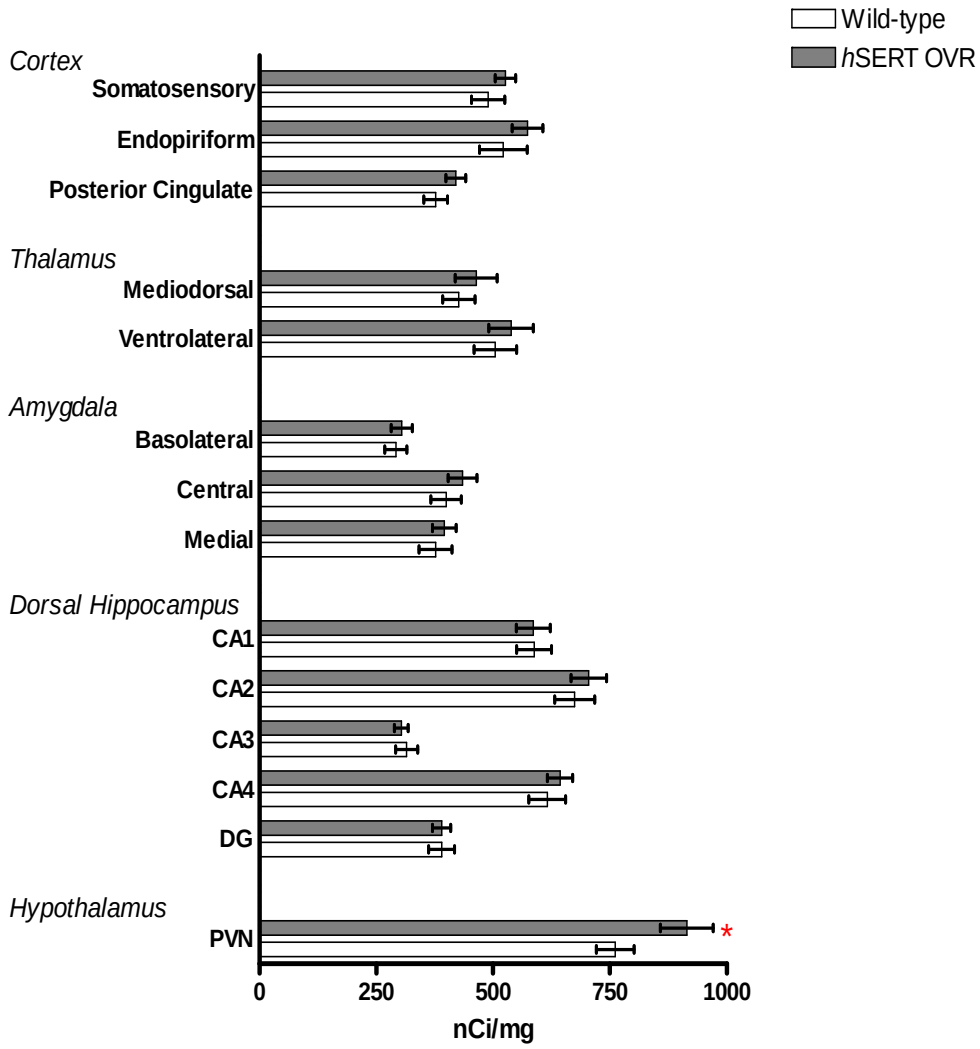
Representative GR and MR mRNA in situ hybridisation autoradiograms from hSERT OVR and Wt male mice. Note the increased MR mRNA levels in the dorsal hippocampus of hSERT OVR mice.

Figure 3.6.4 Effect of *h*SERT OVR on MR mRNA expression



*Effect of hSERT over-expression on mineralocorticoid receptor (MR) mRNA expression (nCi/mg) in the dorsal hippocampus. Data shown as mean ± s.e.m. *denotes p<0.05 significance (Student's t-test). MR mRNA expression is significantly increased in the CA1 and CA2 hippocampal subfields of hSERT OVR mice.*

Figure 3.6.5 Effect of *h*SERT OVR on GR mRNA expression



*Effect of hSERT over-expression on glucocorticoid receptor (GR) mRNA expression. Data shown as mean ± s.e.m. *denotes p<0.05 significant difference from wild-type (Student's t-test). GR mRNA expression is significantly increased only in the hypothalamic paraventricular nucleus of* h*SERT OVR mice.*

Table 3.6.1 Mineralocorticoid receptor mRNA expression in *h*SERT OVR mice

	Wild-type			hSERT OVR			%
	mean		s.e.m	mean		s.e.m	difference
Thalamus							
Mediodorsal	1	±	2	1	±	4	**n/s**
Venterolateral	11	±	3	14	±	1	**28**
Amygdala							
Medial Amygdala	8	±	6	10	±	3	**11**
Basolateral Amygdala	35	±	4	40	±	6	**15**
Central Amygdala	78	±	6	90	±	14	**16**
Dorsal Hippocampus							
CA1	436	±	22	507	±	12	**16***
CA2	365	±	10	442	±	23	**21***
CA3	614	±	15	608	±	39	**-1**
DG	620	±	33	647	±	25	**9**
CA4	289	±	8	315	±	4	**13**

Effect of *h*SERT over-expression on mineralocorticoid receptor (MR) mRNA expression (nCi/mg) in the CNS. Data shown as mean ± s.e.m and % difference between hSERT OVR and wild-type animals. *denotes $p<0.05$ significant difference from wild-type (t-test). n/s denotes no significant expression.

Table 3.6.2 Glucocorticoid receptor mRNA expression in *h*SERT OVR mice

	Wild-type			hSERT OVR			%
	Mean		s.e.m	mean		s.e.m	difference
Hypothalamus							
Paraventricular Nucleus	761	±	40	915	±	52	**20***
Ventromedial Hypothalamus	502	±	32	501	±	49	**0**
Thalamus							
Mediodorsal	427	±	35	464	±	45	**9**
Venterolateral	505	±	45	539	±	48	**7**
Amygdala							
Medial Amygdala	337	±	35	396	±	25	**5**
Basolateral Amygdala	292	±	24	305	±	23	**4**
Central Amygdala	399	±	33	435	±	31	**9**
Dorsal Hippocampus							
CA1	588	±	38	586	±	36	**0**
CA2	675	±	43	705	±	38	**4**
CA3	315	±	23	303	±	14	**-4**
DG	616	±	39	643	±	27	**4**
CA4	390	±	28	390	±	20	**0**
Cortex							
Somatosensory Cortex	489	±	36	526	±	22	**8**
Posterior Cingulate	377	±	26	420	±	21	**11**
Endopiriform	522	±	51	574	±	33	**10**

*Effect of hSERT over-expression on glucocorticoid receptor (GR) mRNA expression (nCi/mg) in the CNS. Data shown as mean ± s.e.m and % difference between hSERT OVR and wild-type animals. *denotes p<0.05 significant difference from wild-type (t-test).*

6.4 Discussions and Conclusions

In this study no evidence was found for an effect of genotype (*h*SERT OVR vs Wt) on HPA axis activity. Circulating ACTH and corticosterone levels were similar in both groups under basal conditions and in response to a mildly stressful event (handling and saline injection). In addition there was no evidence to suggest that the AUC integral in ACTH (Wt: 495, hSERT OVR: 299) and corticosterone (Wt: 1210, hSERT OVR: 1339) secretion from basal levels to those observed 15 minutes after acute saline injection was significantly different between the genotypes. This further supports the suggestion that the acute stress response is not altered in *h*SERT OVR mice. These findings contrast with the reported decrease in basal corticosterone level observed in homozygous SERT KO mice but is in agreement with the finding that basal ACTH levels are not significantly altered in these animals (Li et al., 1999). The reason for the disparities between these opposing models of genetically altered SERT bioavailabilty may directly relate to the magnitude of their respective alterations in SERT function. Indeed, the finding that basal ACTH and corticosterone levels were not altered in heterozygous SERT KO mice, where SERT function is reduced by ~50%, supports the contention that partial depletion of SERT function, as compared to complete loss, may not be sufficient to alter basal HPA axis activity. However, the finding that the ACTH response to acute stress was not reduced in *h*SERT OVR mice is surprising given the reported enhancement of ACTH secretion following acute stress in both heterozygous and homozygous SERT KO mice (Li et al., 1999). In the experiments reported here, however, any difference in ACTH secretion following acute stress between the genotypes may have been masked by the high variability in plasma ACTH concentrations in saline-treated animals. There was also no evidence for altered adrenal sensitivity in *h*SERT OVR mice as the corticosterone : ACTH ratio, under both basal conditions and in animals subjected to acute stress, was not significantly different between *h*SERT OVR (basal 0.52 ± 0.13, stress 0.68 ± 0.17) and Wt (basal 1.48 ± 0.57, stress 1.05 ± 0.22) mice.

No evidence was found to suggest any difference between *h*SERT OVR and Wt mice in the effects on HPA axis activity of manipulating 5-HT_{1A}, 5-$HT_{2A/C}$ or 5-HT_{1B} receptors. Thus genotype had no significant effect upon either ACTH or corticosterone levels following 8-OH-DPAT, DOI or CP 94,253 treatments respectively. In contrast to studies using the same treatment protocol (Li et al., 1999) the present studies found that 8-OH-DPAT treatment failed to significantly increase ACTH levels above that following saline injection. The reason for this disparity is unclear but may relate to differences in the sensitivity of hypothalamic 5-HT_{1A} receptors or the differential activation of these receptors during mild acute stress between different mouse strains. In wild-type animals while 8-OH-DPAT treatment resulted

in no significant alterations in ACTH secretion, however, the level of corticosterone was significantly increased by the drug. This suggests that 8-OH-DPAT may act within the periphery, possibly at the level of the adrenal cortex, to stimulate corticosterone release. This suggestion is consistent with the known affinity of 8-OH-DPAT for the 5-HT_7 receptor and the localisation of this receptor subtype to the adrenal cortex (Contesse et al., 1999), although the physiological role of these receptors in corticosterone secretion is yet to be confirmed. However, it is also important to note that although we found that 8-OH-DPAT did not result in a significant increase in ACTH secretion, numerous other studies have reported this effect in mice (Li et al., 1993; Li et al., 1999; Matsuda et al., 1990). Therefore, it is possible that this mechanism did contribute to the enhanced secretion of corticosterone by 8-OH-DPAT in this study but that this effect was obscured by the high variability in ACTH measurements, especially in saline-treated animals. In either case, these results indicate that 5-HT_{1A} receptor-mediated regulation of HPA axis activity is not altered in *h*SERT OVR mice.

In both genotypes DOI produced a significant increase in ACTH that was similar in magnitude to that reported by others in wild-type animals (Li et al., 2003). While there was a trend towards an increased ACTH response to DOI in *h*SERT OVR mice, which parallels the enhanced LCMRglu response to DOI in these animals (study 4), the effect was not significant. The finding that the ACTH response to DOI was not significantly altered in *h*SERT OVR mice is consistent, however, with the lack of alteration in hypothalamic 5-$HT_{2A/C}$ receptor sensitivity reported in both heterozygous and homozygous SERT KO mice (Li et al., 2003). As expected from the observed alterations in ACTH secretion, DOI also produced a significant increase in circulating corticosterone the magnitude of which was similar in both genotypes.

In contrast to reports in which 5-HT_{1B} agonists have been shown to increase ACTH secretion (Calogero et al., 1990; Vandekar et al., 1994) CP 94,253 treatment did not increase circulating ACTH levels measured in the present study. In fact, CP 94,253 produced a trend towards decreased ACTH levels in animals of both genotypes, although this effect was not significant. While this disparity may be attributed in some instances to the activation of other 5-HT receptor subtypes by non-selective 5-HT_{1B} receptor ligands (mCPP has both 5-HT_{1B} and 5-HT_{1C} affinity (Calogero et al., 1990)) others studies have reported the same effect using more selective drugs (CP 93,129 is highly selective for the 5-HT_{1B} receptor (Van de Kar et al., 1994)). Disparities between the effects on ACTH secretion of the equally selective 5-HT_{1B} agonists CP 93,129 and CP 94,253 may reflect the route of drug administration or subtle differences in the properties of the drugs themselves. In the study by Van de Kar et al. (1994) the administration of CP 93,129 via the intracerebroventricular (*i.c.v*) route is likely to have

avoided the activation of peripheral 5-HT_{1B} receptors, particularly as the drug does not readily cross the blood brain barrier (BBB). In contrast, in the present study the 5-HT_{1B} agonist CP 94,253 was administered *i.p* and as it does readily cross the BBB results in the activation of both central and peripheral 5-HT_{1B} receptors. Thus, the observation that CP 94,253 results in a robust increase in corticosterone secretion without altering ACTH levels suggests that CP 94,253 may be acting in the periphery, possibly at the level of the adrenal cortex, to increase corticosterone levels. Moreover, this peripheral action CP 94,253 may contribute to the observed decrease in ACTH secretion (albeit non-significant) via glucocorticoid-mediated feedback onto the HPA axis. In addition, the increase in ACTH secretion induced by the mild stressor of saline injection may require the activation of post-synaptic 5-HT receptors. In CP 94,253 treated mice ACTH secretion may also be reduced in comparison to saline injected animals through the inhibition of serotonergic neurotransmission by nerve terminal autoreceptors activated by the drug. However, given the evidence that central 5-HT_{1B} receptor activation stimulates rather than attenuates ACTH secretion (Can de Kar et al., 1994) the role of enhanced glucocorticoid-mediated feedback in the influence of CP 94,253 on ACTH secretion seems the more likely mechanism.

Despite the marked alterations in circulating corticosterone levels produced by 8-OH-DPAT, DOI and CP 94,253 there was no evidence that this effect was modified in *h*SERT OVR mice. In addition to the direct importance of this finding with regard to understanding the regulation of HPA axis functioning in *h*SERT OVR mice, it also has important implications in the interpretation of data from the LCMRglu studies reported previously in this thesis, and supports the suggestion that genotype-dependent differences in LCMRglu responses to these drugs cannot be attributed to genotype-dependent effects on circulating corticosterone levels, which themselves are known to influence LCMRglu (Doyle et al., 1994).

Although HPA axis activity (as indicated by measurements of circulating ACTH and corticosterone) was not altered under basal conditions or in response to mild stress in *h*SERT OVR mice, significant alterations in basal MR and GR mRNA expression levels were found. If these were translated directly into similar alterations in levels of receptor protein expression, they might be expected to result in altered HPA axis activity. Specifically, enhanced GR and MR receptor expression in *h*SERT OVR mice may result in enhanced glucocorticoid-mediated negative feedback upon the HPA axis which could attenuate the stress response or alter the circadian rhythm of the axis. This suggestion warrants further systematic investigation.

The expression of MR mRNA was significantly increased in CA1 and CA2 subfields of hippocampus in *h*SERT OVR mice and this may contribute directly to their reduced anxiety phenotype. Certainly enhanced forebrain expression of MR has previously been shown to be associated with reduced anxiety-like behaviour (Lai et al., 2007; Rozeboom et al., 2007). Furthermore, the increased MR mRNA expression observed in *h*SERT OVR mice also parallels that observed following chronic antidepressant treatment (Seckl and Fink, 1992), which also results in reduced anxiety-like behaviour. In contrast, the acute activation of hippocampal MR appears to be anxiogenic (Bitran et al., 1998; Smythe et al., 1997). Taken together, these data suggest that adaptive responses resulting from a prolonged enhancement of hippocampal MR signalling, rather than any acute enhancement, may be necessary to elicit an anxiolytic behavioural phenotype. It is of great interest, therefore, that the present studies show a paradoxical reduction in hippocampal 5-HT_{1A} receptor binding in *h*SERT OVR mice (study 3) despite the reduced synaptic 5-HT levels in these animals which might be expected to result in an up-regulation of post synaptic receptors. As MR signalling in the hippocampus has an inhibitory effect on 5-HT_{1A} receptor expression (Meijer et al., 1997; Meijer and DeKloet, 1995) it may be the prolonged enhancement of MR receptor signalling in hSERT OVR mice that induces the reduced 5-HT_{1A} binding noted in these animals. Futhermore, the interaction between hippocampal MR signalling and 5-HT_{1A} receptor expression may represent an important mechanism by which SERT expression regulates anxiety, as 5-HT_{1A} receptor activation in the hippocampus in anxiogenic (File et al., 1996). Therefore, the possible interaction between hippocampal MR and 5-HT_{1A} receptor signalling in *h*SERT OVR mice, and the role of this interaction in the regulation of anxiety behaviour warrants, further investigation.

It is interesting that the increased MR mRNA expression observed in *h*SERT OVR mice also parallels that observed following chronic SSRI treatment (Bjartmar et al., 2000; Seckl and Fink, 1992; Semont et al., 2000), directly implicating chronic alterations in extracellular 5-HT levels in the control of MR receptor gene expression. Therefore, given that extracellular 5-HT levels are chronically reduced in *h*SERT OVR mice the results of the present studies seem somewhat surprising, especially given that others have found that reduced 5-HT neurotransmission is associated with reduced hippocampal MR mRNA expression (Seckl et al., 1990; Yau et al., 1994). It is likely that developmental alterations may account for the observed enhancement of hippocampal MR mRNA expression in *h*SERT OVR mice despite reduced synaptic 5-HT availability. One possible mechanism may involve a compensatory up-regulation in post-synaptic 5-HT receptor function which might regulate MR mRNA expression. However, while a role has been suggested for 5-HT_7 receptors in the regulation of

hippocampal GR mRNA expression (Laplante et al., 2002) the identity the 5-HT receptor involved in the regulation of hippocampal MR mRNA expression has yet to be elucidated.

Enhanced GR receptor mRNA expression was found to be limited to the PVN of *h*SERT OVR mice. The GR receptor in the PVN is known to play a central role in controlling the activity of the HPA axis at times when circulating levels of glucocorticoids are elevated, for example during stressful events, through a negative feedback mechanism (Feldman et al., 1992; Kovacs et al., 1986). The increased expression in *h*SERT OVR mice might suggests that the amplitude of the stress response or its duration may be attenuated in these animals. The mechanism by which a life-long increase in SERT function is linked to increased PVN GR mRNA expression is unclear, as there is a paucity of data on the regulation of PVN GR expression by 5-HT neurotransmission. However, a number of studies investigating the regulation of GR in hippocampus supports the suggestion that GR gene transcription can be regulated by 5-HT neurotransmission, with 5-HT having a stimulatory effect on GR mRNA expression (Bjartmar et al., 2000; Seckl et al., 1990; Seckl and Fink, 1992). Therefore, not only does it seem surprising that PVN GR mRNA levels are enhanced in *h*SERT OVR mice, where extracellular 5-HT levels are chronically reduced, but the lack of any alteration in hippocampal GR mRNA expression in these mice is also of interest. Compensatory alterations in post-synaptic 5-HT receptor function may again allow for the maintenance of GR mRNA expression in the hippocampus of *h*SERT OVR mice. In future studies attempts to identify possible alterations in the function of hippocampal 5-HT_7 receptors in *h*SERT OVR mice may be of particular interest given the known role of this receptors subtype in the regulation of hippocampal GR mRNA expression (Laplante et al., 2002). Clearly, the relationship between 5-HT neurotransmission and the regulation of PVN GR mRNA expression also warrants further investigation.

Overall these result suggest, therefore, that alterations in basal, or acute stress-induced HPA axis activity associated with the 5-HTTLPR in humans, are unlikely to contribute to differences in the likelihood of developing affective disorders in response to stressful life events between individuals carrying the different polymorphisms. However, it should be noted that measurements of basal ACTH and corticosterone concentrations in the present studies were limited to a point in the circadian cycle at which the circulating concentration of these hormones is particularly low (circadian nadir). It is possible that the basal activity of the HPA axis may be different between *h*SERT OVR and wild-type mice at other points in the circadian cycle, when the concentration of these hormones is higher. This suggestion warrants further investigation given the alterations in MR and GR receptor mRNA levels detected in *h*SERT OVR mice in these studies. Furthermore, these receptor alterations may be expected

to contribute to altered HPA axis activity following stressful events in *h*SERT OVR mice, although such a difference was not detected in this study. One reason for this may be that our measurement of stress-induced HPA axis activation was limited to the initial phase of HPA axis activation (first 15 minutes after stressor) when feed-forward mechanisms contributing to the activity of the HPA axis may predominate. In the future, fuller characterisation of HPA axis activity following a stressful event, across a longer time frame, may reveal differences in the stress response between genotypes. Given the observed differences in GR and MR receptor mRNA expression levels in *h*SERT OVR mice one might expect that the peak amplitude of ACTH/corticosterone secretion or the duration of the stress response may be attenuated in *h*SERT OVR mice, but these were outwith the scope of the present investigation. Furthermore, application of a paradigm associated with a greater level of HPA axis activation (e.g. prolonged restraint stress) may reveal differences in the stress responsiveness of the HPA axis between genotypes. While we found no evidence to suggest that adrenal sensitivity was altered in *h*SERT OVR mice, according to the corticosterone : ACTH ratio in basal and stressed (saline treated) animals,. Observing adrenal size in *h*SERT as compared to wild-type animals may be of interest in the future as this would give an indication of the chronic glucocorticoid status of these animals.

As our previous studies have shown that the alterations in brain function and 5-HT system activity that result from a life-long increase in SERT expression are influenced by gender, with a greater effect being observed in females than in males, it is also likely that any alterations in HPA axis function that results from increased SERT expression will also be more pronounced in females than in males. Therefore, investigating the parameters observed in this study in female animals will be of great interest. This may also be particularly relevant given the known interaction between gender and the 5-HTTLPR in the programming of HPA axis responsiveness by early-life stressful events (Barr et al., 2004a).

In summary, we found no evidence for altered basal HPA axis activity, differential activation of the HPA axis by mild stress, or altered 5-HT_{1A}, 5-HT_{1B} or 5-$HT_{2A/C}$ receptor-mediated regulation of the HPA axis as a result of a life-long increase in SERT expression. This suggest that alterations in the basal or feed-forward drive activity of the HPA axis may not be a central mechanism by which the 5-HTTLPR modifies the risk of affective disorder in response to stressful life events in humans. However, evidence was found suggesting that central MR and GR receptor function may be modified by a life-long increase in SERT functioning. Alterations in these receptors may contribute to the reduced anxiety found in *h*SERT OVR mice, and may modify the activity of the HPA axis during the circadian cycle, or in response to stressful events, although these have yet to be confirmed. Therefore,

differential expression of the GR and MR receptors in response to genetically determined SERT function may play a role in the effect of the 5-HTTLPR on affective functioning, antidepressant response and risk of developing affective disorders in response to stressful life events.

Chapter 4- Final Discussion and Conclusions

1. Summary

The data presented in this thesis have characterised the effects of a life-long, genetically-determined increase in SERT bioavailability upon the pharmacology of the serotonergic system (ligand binding), neuronal activity in response to serotonergic challenge (2-deoxyglucose imaging) and hypothalamo-pituitary-adrenal axis function (hormone analysis and in situ hybridisation). Furthermore, the importance of gender in determining aspects of neuronal function and serotoninergic pharmacology have been examined in order to further elucidate gender differences in general brain function and their interaction with a life-long increase in SERT.

Gender appears to be an important factor and a number of differences have been found when male and female mice are compared. Thus the functional response to 5-HT_{1A} receptor challenge has been found to be enhanced in females, in part as a result of altered receptor binding; 5-HT_{1B} receptor functioning is also enhanced, despite no difference in receptor binding; and 5-$HT_{2A/C}$ receptor function is decreased, in part as a result of altered 5-HT_{2A} and 5-HT_{2C} binding in females as compared to males. Table 4.1 provides a summary of the gender differences in the 5-HT system reported in this thesis, while tables 4.2.1 and 4.2.2 provide a more comprehensive summary of the gender differences in 5-HT receptor mediated functional responses in different functional systems. Evidence for sexually dimorphic alterations in the functional response to serotonergic challenges were particularly prevalent in cortical regions, the basal ganglia, mesocorticolimbic system and the raphé, while these dimorphisms were much more limited in the hippocampus and amygdala nuclei.

Table 4.1. Gender differences in 5-HT system function

	Binding	Function
SERT	**F > M**	
5-HT_{1A}	**F > M**	**F > M**
5-HT_{1B}	**n/s**	**Hypo-stimulated in F**
5-HT_{2A}	**F < M**	**F < M**
5-HT_{2C}	**F < M**[1]	

[1] *sexual-dimorphism limited to nucleus accumbens.*

Table 4.2.1 Gender differences in LCMRglu responses to 5-HT receptor agonists

	Wild-type				*h*SERT OVR				*h*SERT x Gender interaction		
	8-OH-DPAT	DOI	CP		8-OH-DPAT	DOI	CP		8-OH-DPAT	DOI	CP 94,253
Cortex											
Orbitofrontal											
Frontal											
Anterior Cingulate											
Dorsal mPFC											
Ventral mPFC											
Somatosensory											
Temperoparietal											
Posterior Cingulate											
Piriform											
Entorhinal											
Basal Ganglia											
Medial Striatum											
Lateral Striatum											
Globus Pallidus											
Subthalamic nucleus											
Substantia nigra pars reticulata											
Substantia nigra pars compacta											
Amygdala											
Medial											
Basolateral											
Central											
Thalamus											
Anterior											
Mediodorsal											
Venterolateral											

Gender differences in the LCMRglu responses and modification of these differences by hSERT OVR in cortical, basal ganglia, amygdala and thalamic regions. Red denotes significant increase in LCMRglu response in females as compared to males. Blue denotes significant decrease in the LCMRglu response in females as compared to males and the significant attenuation of the gender difference by hSERT OVR (in hSERT OVR x gender interaction column). Significance set at p<0.05 throughout.

Table 4.2.2 Gender differences in LCMRglu responses to 5-HT receptor agonists

	Wild-type			*h*SERT OVR			*h*SERT x gender interaction		
	8-OH-DPAT	DOI	CP 94,253	8-OH-DPAT	DOI	CP 94,253	8-OH-DPAT	DOI	CP 94,253
Hypothalamus									
Anterior									
Venterolateral									
Hippocampus									
Molecular Layer									
Dorsal Subiculum									
Dentate PO									
Dorsal CA1									
CA2									
Ventral CA1									
Ventral Subiculum									
CA3									
Raphè									
Dorsal									
Median									
Paramedian									
Mesocorticolimbic system									
Ventral tegmental area									
Nucleus accumbens									
Non-specific									
Septal nucleus									
BNST									
Lateral Habenula									
Mamillary Body									
Peri-aqueductal Grey									
Inferior Colliculus									
Locus Coeruleus									

Gender differences in the LCMRglu responses and modification of these differences by hSERT OVR in hypothalamic, hippocampal, raphé, mesocorticolimbic and non-specific regions. Red denotes significantly increased LCMRglu response in females as compared to males. Blue denotes significant decrease in the LCMRglu response in females as compared to males or the significant attenuation of the gender differencein the LCMRglu response by hSERT OVR (in hSERT OVR x gender interaction column). Significance set at p<0.05 throughout.

A life-long increase in serotonin transporter function appears to result in a decrease in constitutive neuronal activity in several limbic regions (Figure 4.1). Furthermore, this effect was more pronounced in females when compared to males. In addition, several alterations in neuronal function following pharmacological challenge to the central serotonin system were identified that may contribute to this hypo-metabolism, and to the reduced anxiety behaviour reported in *h*SERT OVR mice (Jennings *et al*., 2006). Specifically, 5-HT_{1A} receptor function was found to be decreased and 5-$HT_{2A/C}$ function increased in response to a life-long increase in SERT. Hypo-activation of the 5-HT_{1B} receptor in female *h*SERT OVR mice appears to be a major contributory factor in the cerebral hypo-metabolism measured in these animals.

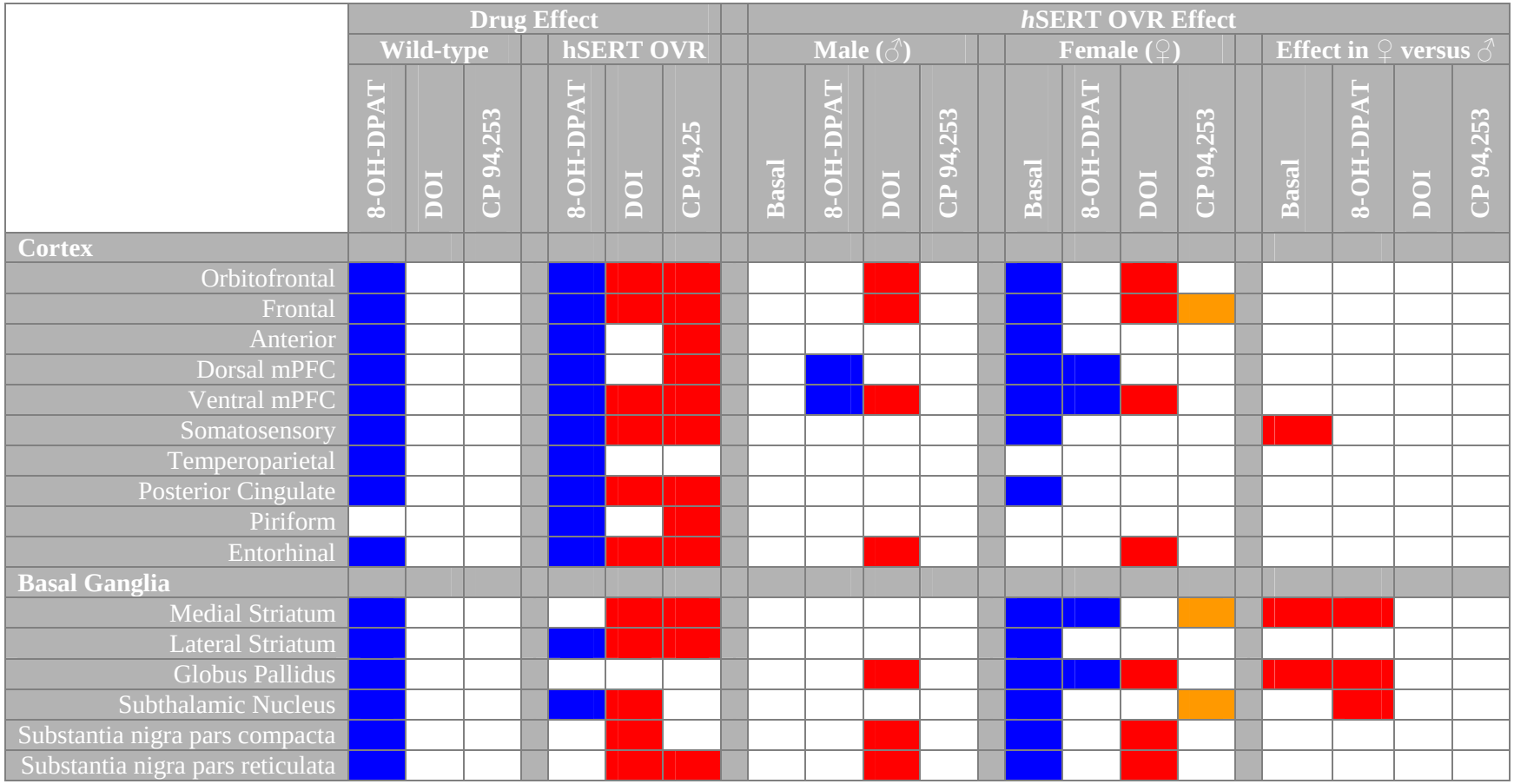
The alterations in neuronal activity of *h*SERT OVR mice following challenges to the serotonin receptors appear to be widespread throughout the brain, occurring in all functional systems. The contribution of altered 5-HT receptor binding levels to observed changes in the functional response to serotonergic challenges in appears to be relatively limited. The most evident alterations in sertonergic receptor binding in *h*SERT OVR mice were a generalised decrease in 5-HT_{1A} and a more localised increase in 5-HT_{2C} receptor binding. However, there was no evidence for any alteration in the binding of the 5-HT_{1B} or 5-HT_{2A} receptors despite clear changes in functional activity in the brain when these receptors were challenged. Table 4.3 provides a summary of the alterations in 5-HT receptor binding and function found in *h*SERT OVR mice, and tables 4.4.1 to 4.4.4 provide a more comprehensive summary of the differences in 5-HT receptor mediated functional responses across the different functional systems.

Table 4.3 *h*SERT OVR differences in 5-HT system function

	Binding	**Function**	**Gender x *h*SERT interaction**
Constitutive		***h*SERT < Wt**	**$F > M^2$**
SERT	***h*SERT > Wt**		**$F > M^1$**
5-HT_{1A}	***h*SERT < Wt**	***h*SERT < Wt**	**$M>F^1$; $F > M^2$**
5-HT_{1B}	***h*SERT = Wt**	**Hypo-stimulated in *h*SERT**	**$F > M^2$**
5-HT_{2A}	***h*SERT = Wt**	***h*SERT > Wt**	***n/s***
5-HT_{2C}	***h*SERT > Wt**		

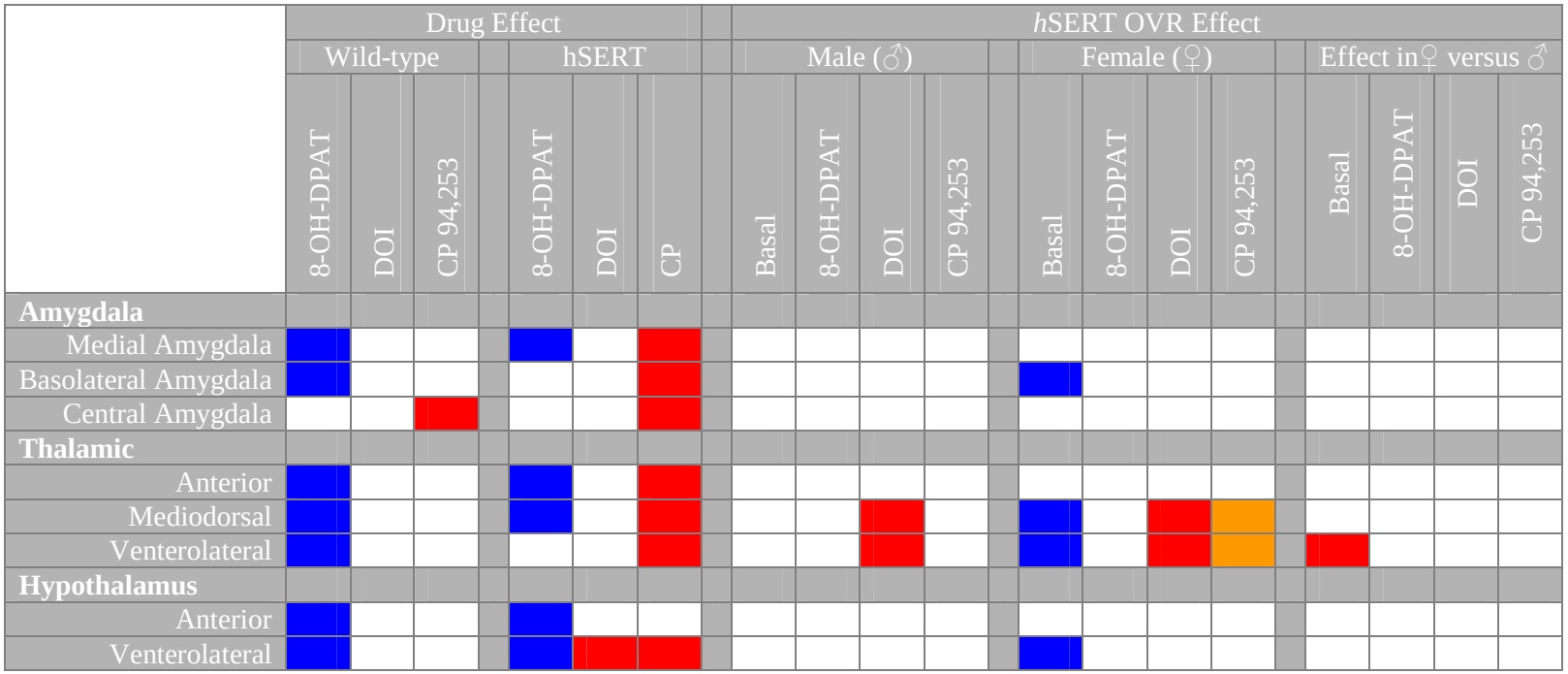
Interaction denotes relative magnitude of alterations between male and female animals.[1]denotes interaction in binding, [2]denotes interaction in function.

Table 4.4.1 Effect of *h*SERT OVR on LCMRglu responses: Cortical and Basal ganglia regions



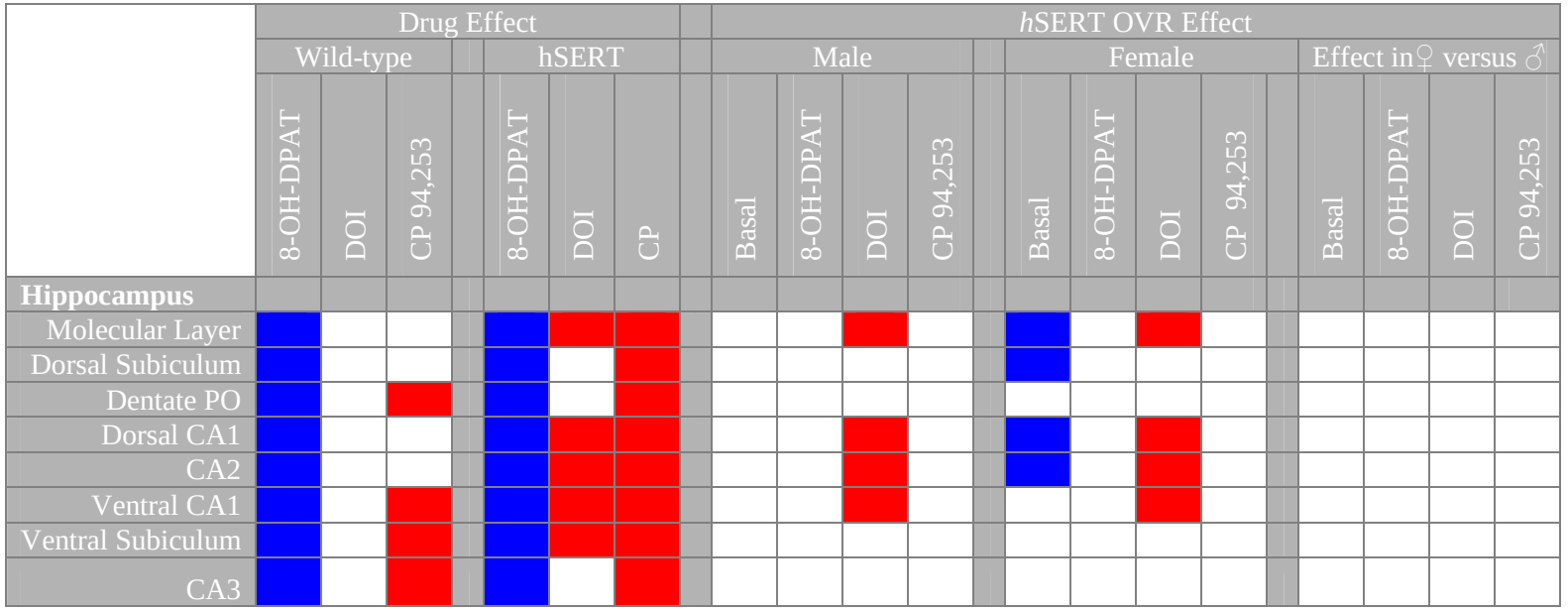
Summary of the influence of hSERT OVR on LCMRglu responses in cortical and basal ganglia regions. Blue denotes a significant decrease in LCMRglu or a significant decrease in the LCMRglu response. Red denotes significant increase in LCMRglu or a significant increase in the LCMRglu response. Orange represents an increase in the LCMRglu response to a drug bringing LCMRglu to wild-type baseline level. Significance set at p<0.05 throughout.

Table 4.4.2 Effect of *h*SERT OVR on LCMRglu responses: amygdala, thalamic and hypothalamic regions



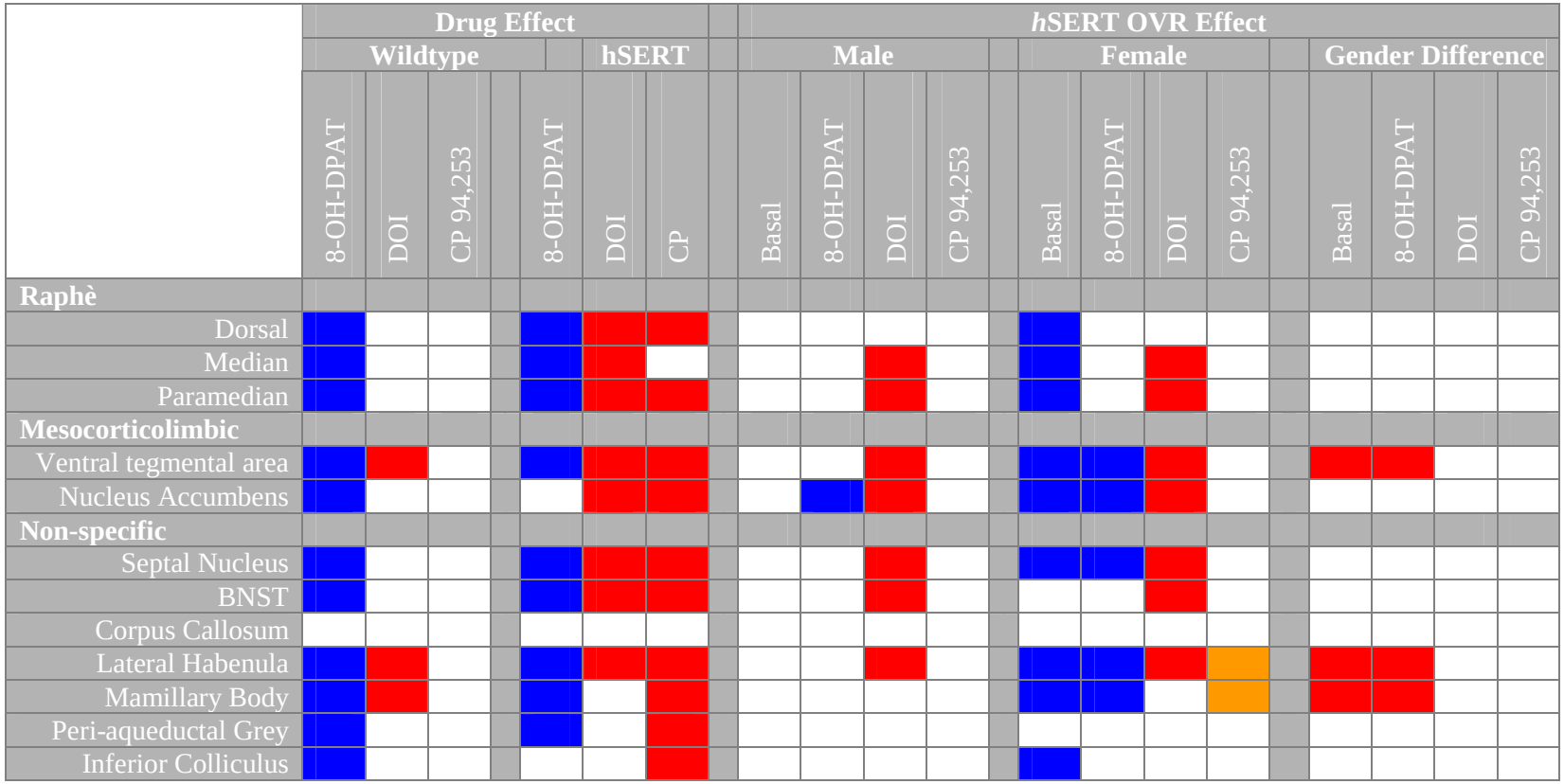
Summary of the influence hSERT OVR on LCMRglu responses in serotonergic challenge in amygdala, thalamic and hypothalamic regions. Blue denotes a significant decrease in LCMRglu or a significant decrease in the LCMRglu response. Red denotes significant increase in LCMRglu or a significant increase in the LCMRglu response. Orange represents an increase in the LCMRglu response to a drug bringing LCMRglu to wild-type baseline level. Significance set at p<0.05 throughout.

Table 4.4.3 Effect of *h*SERT on LCMRglu responses: hippocampal regions



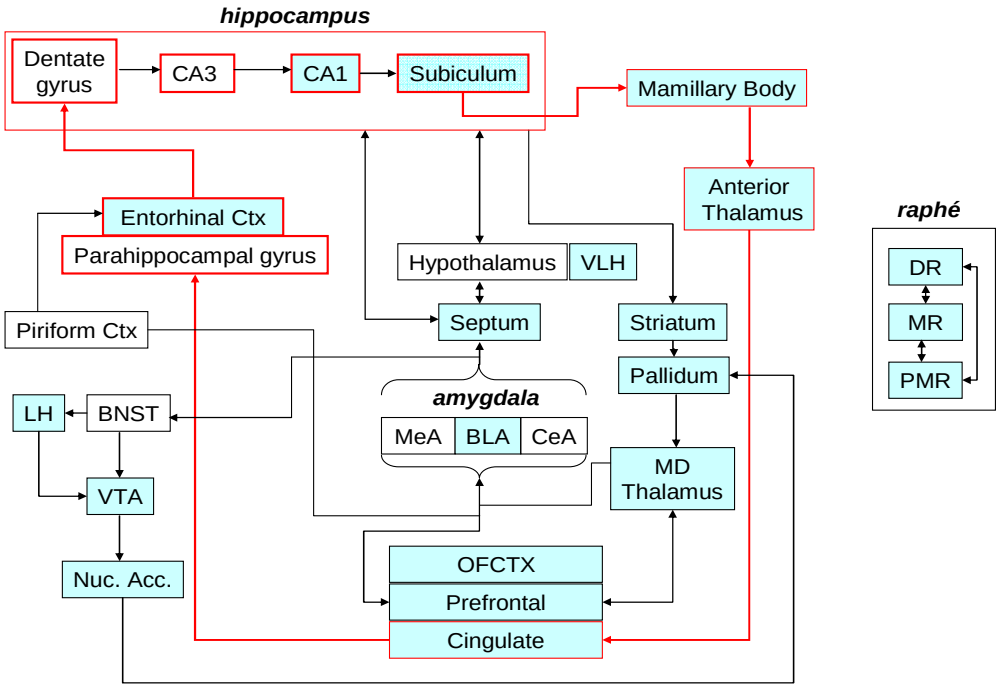
Summary of the influence of hSERT OVR on the LCMRglu response to serotonergic challenge in hippocampal regions. Blue denotes a significant decrease in LCMRglu or a significant decrease in the LCMRglu response. Red denotes significant increase in LCMRglu or a significant increase in the LCMRglu response. Orange represents an increase in the LCMRglu response to a drug bringing LCMRglu to wild-type baseline level. Significance set at p<0.05 throughout.

Table 4.4.4 Effect of *h*SERT on LCMRglu responses: raphé, mesocorticolimbic and non-specific regions



Summary of the influence of hSERT OVR on LCMRglu responses to serotonergic challenge in raphé, mesocorticolimbic and non-specific brain regions. Blue denotes a significant decrease in LCMRglu or a significant decrease in the LCMRglu response. Red denotes significant increase in LCMRglu or a significant increase in the LCMRglu response. Orange represents an increase in the LCMRglu response to a drug bringing LCMRglu to wild-type baseline level. Significance set at $p<0.05$ throughout.

Figure 4.1 Constitutive alterations in limbic system function in *h*SERT OVR mice



hSERT over-expression results in significant constitutive hypometabolism in multiple components of the limbic system. Blue shaded areas represent those regions in which a significant hypometabolism is observed in hSERT OVR female, in which the effect was more pronounced than in males, as compared to wild-type mice. Red outlined regions represent those components outlined in the original Papez circuit, proposed to be involved in the regulation of emotion (Papez, 1937). Other components included in the diagram are also known to be involved in the regulation of emotion and are components of the "limbic system" (for a review see Rajmohan and Mohandas., 2007). The raphé nuclei innvervate all components of the limbic system. Abbreviations: BLA: Basolateral amygdala, BNST: Bed Nucleus of the Stria Terminalis, CeA: Central Amygdala, DR: Dorsal raphé, Entorhinal Ctx: Entorhinal cortex, LH; Lateral Habenula, MeA: Medial Amygdala, MD Thalamus: Mediodorsal Thalamus, MR: Median Raphé, Nuc. Acc.: Nucleus Accumbens,OFCTX: Orbitofrontal Cortex, Piriform Ctx: Piriform Cortex, PMR: Paramedian raphé, VLH: venterolateral hypothalamus, VTA: Ventral Tegmental Area.

This thesis provides clear evidence to suggest that the effects of altered SERT bioavailability upon brain function and serotonergic pharmacology are more extensive in female mice than in males. A major contributory mechanism to this is likely to involve the greater enhancement of SERT protein expression in female *h*SERT OVR mice, as shown by radioligand binding, despite an identical SERT genotype between the genders. The effects of a life-long increase in SERT upon constitutive cerebral function were also more pronounced in females than in males. These differences were noted in discrete components of multiple functional systems including the ventral tegmental area, thalamic nuclei, components of the basal ganglia, the lateral habenula and mammillary body. Gender-dependent modulation of the alterations in SERT protein expression, 5-HT_{1A} receptor function and the degree of 5-HT_{1B} receptor hypo-activation as a result of a genetically driven increase in SERT gene expression may be particularly important in determining this gender difference in brain function.

Measurements of circulating levels of corticosterone and ACTH provided no evidence for altered basal activity, stress-induced activation or serotonin receptor mediated regulation of HPA axis function in male *h*SERT OVR. There were, however, profound alterations in hypothalamic GR and hippocampal MR mRNA expression levels which are suggestive of enhanced negative-feedback regulation of the HPA axis in *h*SERT OVR mice.

2. Relevance of Present Work

The increased susceptibility of human females to the development of affective disorders, may relate to some of the sexual dimorphisms identified in both genotypes in these studies, many of which are consistent with a relative serotonergic hypo-function in females as compared to males. This is consistent with previous findings of decreased 5-HT turnover in human females (Nishizawa et al., 1997) and decreased extracellular 5-HT levels the forebrain of female rodents (Gundlah et al., 1998; Jones and Lucki, 2005) when compared to males. Such gender-related differences would be expected to add to the decreased post-synaptic 5-$HT_{2A/C}$ receptor binding and function found in these studies, making it even more likely that serotonergic hypo-function would be generated in females. Furthermore, the increased 5-HT_{1A} autoreceptor binding and function in females, found both here and by others (Bouali et al., 2003; Maswood et al., 1995), suggests that enhanced 5-HT_{1A} autoreceptor-mediated inhibition of 5-HT neuronal activity may also contribute to this proposed serotonergic hypo-function in females. The decreased availability of forebrain

extracellular 5-HT in females appears to be compensated for by an up-regulation in post-synaptic 5-HT_{1A} binding and so this serotonergic hypo-function is unlikely to involve this receptor mechanism.

However, while in male mice pharmacological challenge aimed at the 5-HT_{1B} receptor has no effect upon brain function, suggesting that these receptors appear to be tonically activated under basal conditions, the same challenge produces marked increases in functional activity in female mice. This observation further supports the idea of serotonergic hypo-function in females.

These 5-HT receptor differences are not only likely to contribute to an enhanced risk of affective disorders in females but may also contribute to the decreased efficacy of antidepressant treatment. Antidepressant (SSRI) treatment produces an increase in extracellular 5-HT that is self-limited through feedback activation of 5-HT_{1A} autoreceptors. Therefore, gender differences in the balance of 5-HT receptor function found in these studies support the contention that the effects of SSRI treatment on neuronal functioning will be smaller in females than in males. For example, it might be expected that the increased sensitivity of 5-HT_{1A} autoreceptors identified here could present a mechanism by which the increase in extracellular 5-HT following SSRI treatment will be more limited in females. Furthermore, post-synaptic 5-HT receptor functioning (e.g. 5-$HT_{2A/C}$) is also lower in females, providing a further mechanism to restrict the post-synaptic response during SSRI treatment.

Despite uncertainties in the literature the overwhelming body of evidence implicates a role for genetically-determined SERT function in the aetiology of affective disorders and the response to antidepressants in humans (see introduction section 6.3). The work presented here has identified profound alterations in cerebral serotonergic systems and HPA axis functioning as a result of a life-long increase in SERT bioavailability. These alterations may provide the mechanism by which genetically altered SERT levels influence affective functioning and the antidepressant response. Furthermore, the observation that the effects of a life-long increase in SERT function are greater in females than in males is consistent with the finding that the 5-HTTLPR polymorphism has a greater effect on affective functioning (Brummett et al., 2003; Du et al., 2000) in human females as compared to males.

While extracellular levels of 5-HT are likely to be lower in 'L' as compared to 'S' allele individuals, as a result of enhanced SERT function, our results suggest that compensatory alterations in 5-HT receptor function are likely to occur. In particular, 5-HT_{1A} autoreceptor function is likely to be decreased whereas post-synaptic 5-$HT_{2A/C}$ receptor function is likely to be enhanced. However, it would appear that the compensatory increase in 5-HT neuronal activity resulting from 5-HT_{1A} autoreceptor down-regulation is insufficient to maintain the same level of extracellular 5-HT availability in response to a lifelong increase in SERT functioning.

While our data suggest that compensatory alterations in the function of some post-synaptic (e.g. 5-$HT_{2A/C}$) receptors occur in response to increased SERT bioavailabilty a paradoxical decrease in post-synaptic 5-HT_{1A} receptor expression was found in the hippocampus. This paradoxical alteration in hippocampal 5-HT_{1A} receptor expression may be a response to the increased MR function in this region of *h*SERT OVR mice, rather than a result of altered extracellular 5-HT levels. Furthermore, decreased hippocampal 5-HT_{1A} activity may directly contribute to the reduced anxiety in 'L' as compared to 'S' 5-HTTLPR individuals, as it has been shown that hippocampal 5-HT_{1A} receptor activation increases anxiety-like behaviour in animal models (File et al., 1996). While this mechanism may be primary in the regulatory influence of the 5-HTTLPR in males, our studies suggest that other mechanisms may also have a role in females. In particular, the hypo-stimulation of the 5-HT_{1B} receptor subtype in females appears to be directly responsible for the constitutive decrease in neuronal activity in the limbic system of these animals. Therefore, it may be plausible that in humans the 5-HTTLPR mediates its effects on anxiety in males and females via divergent mechanisms.

The increased efficacy of SSRI antidepressant treatment in humans with the 'L' as compared to the 'S' variant of the 5-HTTLPR may relate to the differential sensitivity of the 5-HT_{1A} autoreceptors between these genotypes. This is supported by the findings in this thesis (Study 3) and by those of others (Jennings et al., 2006). The desensitisation of 5-HT_{1A} autoreceptors during chronic SSRI treatment has been proposed to have a central role in determining the efficacy of these drugs in depression. When SSRIs are administered acutely the blockade of SERT results in increased levels of extracellular 5-HT. However, the concomitant activation of 5-HT_{1A} autoreceptors by this acute increase in extracellular 5-HT levels inhibits the activity of 5-HT neurones, limiting any rise in extracellular 5-HT (Gartside et al., 1995; Malagie et al., 1996). During chronic SSRI treatment, in contrast, the down-regulation of 5-HT_{1A} autoreceptor function is proposed to alter the balance between 5-

HT neuronal activity and SERT blockade, resulting in the increase of extracellular 5-HT levels that is the purpose of the therapy (Kantor et al., 2001). It is at this point that SSRIs are proposed to begin demonstrating their anti-depressant action in depressed patients (Hjorth et al., 2000). If the decreased sensitivity of 5-HT_{1A} autoreceptors exists as a result of the life-long increase in SERT function in human individuals with the 'L' allele, one may expect that the balance between SERT blockade and 5-HT_{1A} autoreceptor activation during SSRI treatment is altered in such a way as to allow an even greater enhancement of extracellular 5-HT. Alternatively, the balance between 5-HT_{1A} autoreceptor function and SERT blockade following SSRI administration may mean that the activity of 5-HT neurones in 'L' allele human individuals is closer to the threshold at which extracellular 5-HT is therapeutically increased following SSRI treatment. Therefore, a smaller degree of 5-HT_{1A} autoreceptor down-regulation may be required in 'L' allele individuals in order to achieve enhanced, therapeutic extracellular 5-HT levels. This suggestion would be consistent with the finding that the delay in the onset of antidepressant efficacy is reduced in 'L' allele individuals with depression (Serretti et al., 2007). In humans the administration of 5-HT_{1A} receptor antagonists has been found to increase the efficacy of SSRI treatment in depression, presumably by blocking the SSRI-induced activation of 5-HT_{1A} autoreceptors (Artigas et al., 1996; Olver et al., 2000). Therefore, our results may suggest that this form of dual treatment may be particularly effective at increasing the efficacy of SSRIs in 'S' allele human individuals. The enhanced function of 5-HT_{1A} autoreceptors in 'S' allele human individuals may also contribute to a serotonergic hypo-function that would be consistent with the increased risk of depressive disorders in these individuals.

Due to the inefficacy of conventional antidepressants (SSRIs and TCAs) in the treatment of affective disorders in some patients and the delay in the onset of their therapeutic effects alternative pharmacological treatments are being sought. Many of these new treatments target components of the serotonin system other than SERT, including some of the 5-HT receptors investigated in this thesis. The altered functioning of 5-HT receptors by a life-long increase in SERT functioning observed in this study suggests that humans with the different variants of the 5-HTTLPR may display differential sensitivity to these future therapies. As previously noted, our data suggest that humans with the 'S' allele, whom it is proposed have enhanced 5-HT_{1A} autoreceptor function, may represent good candidates for the augmentation of SSRI treatment by 5-HT_{1A} autoreceptor antagonists, but this approach is unlikely to be as effective in 'L' allele individuals. Furthermore, in our studies we also found that 5-$HT_{2A/C}$ mediated functional effects were enhanced in response to a life-long increase in SERT

function. At present a number of 5-$HT_{2A/C}$ antagonists have been found to decrease anxiety-like behaviour in preclinical models (Griebel et al., 1997). If 5-$HT_{2A/C}$ receptor antagonists ever become used as anxiolytics in a clinical context the efficacy of these drugs may be influenced by the 5-HTTLPR. Overall, these data suggest that it may be imperative for clinical studies to separate patient samples on the basis of the 5-HTTLPR when assessing the efficacy of novel therapeutic agents targeting 5-HT receptors.

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Appendix 1 - Plasma Variables from [^{14}C]-2-Deoxyglucose Experiments

Table A1.1 Plasma variables in constitutive [^{14}C]-2-deoxyglucose experiments

	Male		Female	
	Wt	***h*SERT OVR**	**Wt**	***h*SERT OVR**
Plasma Glucose (mg/ml)	9.03 ± 0.61	7.28 ± 0.80	6.52 ± 0.44*	7.68 ± 0.49
Plasma [^{14}C] (nCi/ml)	77.47 ± 4.53	75.69 ± 8.55	96.05 ± 13.57*	138.48 ± 18.12*
Plasma [^{14}C]:[glucose] ratio (nCi/mM)	8.41 ± 0.65	11.89 ± 1.95	15.34 ± 2.11*	18.02 ± 2.41**

*Plasma variables in Wt and hSERT OVR mice from constitutive [^{14}C]-2-deoxyglucose experiments. Data expressed as mean ± s.e.m. *denotes p<0.05, **denotes p<0.01 significant difference from males of same genotype (t-test with Bonferroni correction). There was no significant effect of hSERT OVR on any plasma variable in either male of female animals.*

Table A1.2 Plasma variables in mice from 8-OH-DPAT [^{14}C]-2-deoxyglucose experiments

	Male			
	Saline		8-OH-DPAT	
	Wt	*h*SERT OVR	Wt	*h*SERT OVR
Glucose (mg/ml)	9.03 ± 0.61	7.28 ± 0.80	6.74 ± 0.40*	4.76 ± 0.28*
[^{14}C] (nCi/ml)	77.47 ± 4.53	75.69 ± 8.55	53.64 ± 4.39	32.2 ± 3.94*
[^{14}C]:[glucose] ratio (nCi/mg)	8.41 ± 0.65	11.89 ± 1.95	8.05 ± 0.73	6.67 ± 0.73*

	Female			
	Saline		8-OH-DPAT	
	Wt	*h*SERT OVR	Wt	*h*SERT OVR
Glucose (mg/ml)	6.52 ± 0.44	7.68 ± 0.49	7.89 ± 0.31*	7.51 ± 0.45
[^{14}C] (nCi/ml)	96.05 ± 13.57	138.48 ± 18.12	53.49 ± 9.06*	68.04 ± 9.16*
[^{14}C]:[glucose] ratio (nCi/mg)	15.34 ± 2.11	18.02 ± 2.41	5.66 ± 1.12**	9.75 ± 1.54 **

*Plasma data from male and female hSERT over-expressing and wild-type mice from LCMRglu response to 8-OH-DPAT study. *denotes p<0.05, **denotes p<0.01 significant difference from appropriate saline control (Student's t-test).*

Table A1.3 Plasma variables in mice from DOI [^{14}C]-2-deoxyglucose experiments

	Male			
	Wild-type		hSERT OVR	
	Saline	DOI	Saline	DOI
[^{14}C] (nCi/ml)	71.2 ± 5.4	76.4 ± 9.6	71.2 ± 8.6	67.6 ± 9.1
Glucose (mg/ml)	9.4 ± 1.1	8.2 ± 1.2	8.2 ± 1.0	6.17 ± 0.7
[^{14}C]:[glucose] ratio (nCi/mg)	8.2 ± 0.6	9.6 ± 0.8	10.0 ± 1.6	11.1 ± 1.3

	Female			
	Wild-type		hSERT OVR	
	Saline	DOI	Saline	DOI
[^{14}C] (nCi/ml)	96.0 ± 13.5	117.2 ± 22.3	154.6 ± 25.5	143.5 ± 21.2
Glucose (mg/ml)	6.5 ± 0.4	7.4 ± 0.4	7.7 ± 0.5	7.68 ± 0.5
[^{14}C]:[glucose] ratio (nCi/mg)	14.8 ± 2.0	16.8 ± 3.8	19.9 ± 2.5	19.9 ± 2.5

Plasma data from male and female hSERT OVR and Wt mice from LCMRglu response to DOI study. There was no significant difference in any variable between DOI-treated animals and the appropriate saline control.

Table A1.4 Plasma variables from animals in CP 94,253 [^{14}C]-2-deoxyglucose experiments

	Male			
	Saline		8-OH-DPAT	
	Wt	*h*SERT OVR	Wt	*h*SERT OVR
Glucose (mg/ml)	9.03 ± 0.61	7.28 ± 0.80	7.56 ± 0.58	6.03 ± 0.23
[^{14}C] (nCi/ml)	77.47 ± 4.53	75.69 ± 8.55	52.32 ± 5.89	42.96 ± 2.80
[^{14}C]:[glucose] ratio (nCi/mg)	8.41 ± 0.65	11.89 ± 1.95	7.21 ± 0.93	7.20 ± 0.53

	Female			
	Saline		8-OH-DPAT	
	Wt	*h*SERT OVR	Wt	*h*SERT OVR
Glucose (mg/ml)	6.52 ± 0.44	7.68 ± 0.49	6.56 ± 0.21	6.61 ± 0.42
[^{14}C] (nCi/ml)	96.05 ± 13.57	138.48 ± 18.12	130.62 ± 14.52	143.52 ± 21.17
[^{14}C]:[glucose] ratio (nCi/mg)	15.34 ± 2.11	18.02 ± 2.41	20.22 ± 2.53	22.74 ± 3.83

Plasma data from hSERT over-expressing and wild-type animals in CP 94,253 LCMRglu study. Data shown as mean ± s.e.m. There was no evidence for a signficiant difference in any plasma variable between any of the different exprerimental groups (t-test).

Publications Arising from Thesis

ALTERED CEREBRAL GLUCOSE METABOLISM IN THE RESPONSE TO DOI IN SEROTONIN TRANSPORTER OVER-EXPRESSING MICE

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Genetic variation leading to altered serotonin transporter (SERT) expression is a predisposing factor in mood disorders (Pezawas *et al*., 2005). Previous studies have used a novel strain of genetically engineered mice, which over-express human SERT (Jennings et al., 2003), to explore the role of the transporter in determining serotonergic neuronal function. In this study we have used the same transgenic strain and applied the 2-deoxyglucose autoradiographic imaging technique to further investigate altered postsynaptic 5-HT_2 receptor function in these animals when challenged with DOI.

Wild-type and transgenic, CBAxC57Bl6, male mice (27g-39g) were injected *i.p.* with DOI (25mg.kg^{-1}, n = 7 from each genotype) or saline (wild-type, n = 11; transgenic, n = 12). Local cerebral glucose utilisation (LCMRglu) was measured 15 min. later by semi-quantitative [^{14}C]-2-deoxyglucose autoradiography in 48 brain regions of interest (ROI) (Kelly et al., 2002). Data (mean ± s.e.m) were analysed using t-test with Bonferroni correction ($p<0.05$).

Following acute saline there was no significant difference in LCMRglu, in any of the 48 ROI observed, between wild-type and transgenic animals. In wild type animals DOI produced widespread increases in LCMRglu (range 11% to 89%), which because of high intrinsic variability were significantly different from control in only 6 of the 48 regions analysed. In contrast, there was no evidence for increased LCMRglu in DOI-treated transgenic mice. Indeed, in 5 brain regions DOI produced significant decreases in LCMRglu. Only one region, the lateral habenula, showed a non-significant increase in LCMRglu (20%). Following DOI, LCMRglu was consistently lower in transgenic animals when compared to wild type animals in every ROI analysed (range -22% to -50%), and was significantly lower in 15 of the 48 ROI observed.

These results suggest that a life-long increase in SERT expression alters the normal functional response of the endogenous serotonin systems to the 5-HT_2 agonist DOI. A decrease in 5-HT_2 functional activity is suggested, as LCMRglu alterations are attenuated, in SERT over-expressing mice.

Jennings, K. *et al*. (2003) *Br. J. Pharmacol*. **138**, 176P.
Kelly, S. *et al.* (2002). *European Journal of Neuroscience* **15**, 945-952.
Pezawas, L. *et al* (2005) *Nature Neurosci.* **8**, 828-834.
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EVIDENCE FOR ALTERED 5-HT_2, BUT NOT 5-HT_{1A}, RECEPTOR FUNCTION IN MICE OVER-EXPRESSING THE HUMAN SEROTONIN TRANSPORTER

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Polymorphic variation in the human serotonin transporter (hSERT) gene, influencing SERT expression, is a predisposing factor in mood disorders (Pezawas *et al*., 2005). Mice over-expressing hSERT have been used to explore the role of SERT in determining serotonergic neuronal function (Jennings *et al* 2003). Here we examine the effects of selective agonists for $5HT_{1A}$ (8-OH-DPAT) and $5HT_2$ (DOI) receptors upon local cerebral glucose utilisation (LCMRglu) in this transgenic strain.

Wild-type (Wt) and transgenic (Tg) (CBAxC57Bl6, male, 25g-39g) mice were injected *i.p.* with saline (n=11 Wt, 12 Tg), DOI (25mg.kg^{-1}, both n=7) or 8-OH-DPAT (10mg.kg^{-1}, n=8 Wt, 7 Tg). LCMRglu was measured (15min post-DOI; 10min post-8-OH-DPAT) by semi-quantitative [^{14}C]-2-deoxyglucose imaging in 48 brain regions of interest (ROI). Data (mean ± s.e.m) were analysed using t-test with Bonferroni correction ($p<0.05$).

Following acute saline there was no significant difference in LCMRglu between Wt and Tg animals. In Wt animals 8-OH-DPAT produced 8 significant increases in LCMRglu, with increases (5%-78%) evident in every ROI. In Tg animals 8-OH-DPAT produced a larger number (19) of significant increases, with increases (3%-75%) evident in all ROI, but comparison of Wt and Tg animals revealed no significant difference in the LCMRglu response to 8-OH-DPAT.

In Wt animals DOI produced significant increases in LCMRglu in 6 ROI, with increases (11%-89%) observed in all ROI. However, no significant increases in LCMRglu occurred in DOI-treated Tg mice. In fact, DOI produced significant decreases (-33% to -44%) in 5 ROI and LCMRglu was lower in Tg animals than Wt animals in every region (–22% to -50%) being significantly lower in 15 ROI .
These results suggest that a life-long increase in SERT expression alters 5-HT_2 but not 5-HT_{1A} receptor function.

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Pezawas, L. *et al* (2005) *Nature Neurosci.* **8**, 828-34

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THE ACUTE CEREBRAL METABOLIC RESPONSE TO SEROTONIN TRANSPORTER TARGETING DRUGS IS ALTERED IN MICE OVER-EXPRESSING THE HUMAN SEROTONIN TRANSPORTER

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Polymorphic variation in the human serotonin transporter (hSERT) gene alters SERT expression and the efficacy of antidepressant drugs (Smeraldi et al., 1998), although the exact causal linkage is unclear. In this study we have examined the acute effects of drugs targeted at SERT (citalopram and MDMA) in hSERT over-expressing mice (Jennings et al., 2003) in order to determine the link between SERT and general brain function as reflected by local cerebral glucose utilisation (LCMRglu).

Wild-type (Wt) and transgenic (Tg)(CBAxC57Bl6, male, 25g-38g) mice were injected *i.p.* with saline (n=11 Wt, 12 Tg), citalopram ($10mg.kg^{-1}$, n=6 Wt, 8 Tg) or MDMA ($30mg.kg^{-1}$, n=8 Wt, 10 Tg). LCMRglu was measured (10min post-citalopram; 15min post-MDMA) by semi-quantitative [^{14}C]-2-deoxyglucose imaging in 48 brain regions of interest (ROI). Data (mean ± s.e.m) were analysed using 2-way-ANOVA with Bonferroni correction ($p<0.05$).

There was no significant difference in LCMRglu between saline-treated Wt and Tg animals. Despite a trend toward increased LCMRglu in citalopram-treated Wt mice (max 38%) there was no significant difference from control. However, in Tg mice citalopram produced significant increases in 29 ROI (max 70%), although no significant drug x transgene interaction was found for any ROI.

Despite trends for both increases (max 22%) and decreases (max -20%) in MDMA-treated Wt mice there was no significant difference from control. In Tg mice, however, MDMA produced 27 significant increases (max 81%) and no decreases in LCMRglu. The LCMRglu response to MDMA was consistently higher in Tg compared to Wt mice (5%-38%), with a significant drug x transgene interaction in 7 ROI.

These results suggest that a life-long increase in SERT expression alters the functional response to drugs which acutely alter SERT activity.

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Jennings K, *et al.* (2003) Br. J. Pharmacol. **138,**176P.

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HIPPOCAMPAL 5-HT_{1A} RECEPTOR BINDING IS DECREASED IN MICE OVER-EXPRESSING THE HUMAN SEROTONIN TRANSPORTER

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Polymorphic variation in the human serotonin transporter (hSERT) gene, influencing SERT expression, is a predisposing factor in mood disorders (Pezawas et al., 2005). Previous studies using a novel strain of genetically engineered mice, which over-express hSERT, suggest that 5-HT_{1A} receptor function may be altered in these animals (Jennings et al., 2004). Here we use the same strain of mice to further investigate the effect of increased SERT expression on 5-HT_{1A} receptor pharmacology using [^{3}H]WAY 100,635 radioligand binding autoradiography.

5-HT_{1A} receptor binding was characterised in 6 wild-type (Wt) and 6 transgenic (Tg) mice (CBAxC57Bl6 background, male, 25g-39g) using 3nM [^{3}H]WAY100,635 (Preece et al., 2004). Binding was measured in 48 brain regions of interest (ROI) and data (mean ± s.e.m) were analysed using t-test ($p<0.05$).

[^{3}H]WAY100,635 binding was significantly decreased in subfields of the dorsal (CA3, -16%; dentate gyrus, -30%) and ventral (dentate PO,-19%) hippocampus in Tg compared to Wt animals. Binding was not significantly different between Tg and Wt animals in any other ROI.

These results suggest that a life-long increase in SERT expression alters 5-HT_{1A} binding in specific hippocampal subfields. Our data also suggest that the previously reported alteration in 5-HT_{1A} autoreceptor function (Jennings et al., 2004) in these animals is not due to altered 5-HT_{1A} autoreceptor pharmacology, as 5-HT_{1A} binding was unaltered in the raphé of these animals.

Jennings, K. *et al* (2004) *Proc. of the BPS (pA2online)* **2**, 010P
Pezawas, L. *et al* (2005) *Nature Neurosci.* **8**, 828-834.
Preece, M.A. et al (2004) Neuroscience **123**, 725-732.

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